This report presents the recommendations of a WHO expert committee commissioned to coordinate activities leading to the adoption of international recommendations for the production and control of vaccines and other biologicals and the establishment of international biological reference materials.

The report starts with a discussion of general issues brought to the attention of the Committee and provides information on the status and development of reference materials for various antibodies, antigens, blood products and related substances, cytokines, growth factors, endocrinological substances and in vitro diagnostic devices. The second part of the report, of particular relevance to manufacturers and national regulatory authorities, contains revised WHO Recommendations for evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products, for production and control of hepatitis B vaccines and for production and control of yellow fever vaccines. New WHO Guidelines on the independent lot release of vaccines are also included. Finally, there is an update to the procedure for the prequalification of vaccines.

Also included are lists of Recommendations, Guidelines and other documents related to the manufacture and control of biological substances used in medicine, and of International Standards and Reference Reagents for biological substances.
The World Health Organization was established in 1948 as a specialized agency of the United Nations serving as the directing and coordinating authority for international health matters and public health. One of WHO’s constitutional functions is to provide objective and reliable information and advice in the field of human health, a responsibility that it fulfills in part through its extensive programme of publications.

The Organization seeks through its publications to support national health strategies and address the most pressing public health concerns of populations around the world. To respond to the needs of Member States at all levels of development, WHO publishes practical manuals, handbooks and training material for specific categories of health workers; internationally applicable guidelines and standards; reviews and analyses of health policies, programmes and research; and state-of-the-art consensus reports that offer technical advice and recommendations for decision-makers. These books are closely tied to the Organization’s priority activities, encompassing disease prevention and control, the development of equitable health systems based on primary health care, and health promotion for individuals and communities. Progress towards better health for all also demands the global dissemination and exchange of information that draws on the knowledge and experience of all WHO’s Member countries and the collaboration of world leaders in public health and the biomedical sciences.

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The WHO Technical Report Series makes available the findings of various international groups of experts that provide WHO with the latest scientific and technical advice on a broad range of medical and public health subjects. Members of such expert groups serve without remuneration in their personal capacities rather than as representatives of governments or other bodies; their views do not necessarily reflect the decisions or the stated policy of WHO.

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SELECTED WHO PUBLICATIONS OF RELATED INTEREST

WHO Expert Committee on Biological Standardization
Sixtieth report.
WHO Technical Report Series, No. 977, 2013 (viii + 231 pages)
Web site: www.who.int/biologicals

WHO Expert Committee on Biological Standardization
Fifty-ninth report.
WHO Technical Report Series, No. 964, 2012 (viii + 228 pages)

WHO Expert Committee on Biological Standardization
Fifty-eighth report.
WHO Technical Report Series, No. 963, 2011 (viii + 244 pages)

WHO Expert Committee on Biological Standardization
Fifty-seventh report.
WHO Technical Report Series, No. 962, 2011 (viii + 206 pages)

WHO Expert Committee on Biological Standardization
Fifty-sixth report.
WHO Technical Report Series, No. 941, 2007 (x + 340 pages)

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WHO Expert Committee on Biological Standardization

Sixty-first report

This report contains the collective views of an international group of experts and does not necessarily represent the decisions or the stated policy of the World Health Organization.
# Contents

## Abbreviations

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>x</td>
</tr>
</tbody>
</table>

## 1. Introduction

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
</tr>
</tbody>
</table>

## 2. General

### 2.1 Current directions

<table>
<thead>
<tr>
<th>Subsection</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1.1 Strategic directions in biological standardization</td>
<td>3</td>
</tr>
<tr>
<td>2.1.2 Vaccines and biological therapeutics: recent and planned activities</td>
<td>3</td>
</tr>
<tr>
<td>in biological standardization</td>
<td>3</td>
</tr>
<tr>
<td>2.1.3 Blood products and related in vitro diagnostics: recent and planned</td>
<td>4</td>
</tr>
<tr>
<td>activities in biological standardization</td>
<td></td>
</tr>
</tbody>
</table>

### 2.2 Reports

<table>
<thead>
<tr>
<th>Subsection</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2.1 WHO Blood Regulators Network</td>
<td>5</td>
</tr>
<tr>
<td>2.2.2 Reports from the WHO International laboratories and collaborating</td>
<td>5</td>
</tr>
<tr>
<td>centres for biological standards</td>
<td></td>
</tr>
</tbody>
</table>

### 2.3 Issues

<table>
<thead>
<tr>
<th>Subsection</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.3.1 Scientific issues identified by users of WHO biological reference</td>
<td>7</td>
</tr>
<tr>
<td>preparations</td>
<td></td>
</tr>
<tr>
<td>2.3.2 Issues shared with the WHO Expert Committee on Specifications for</td>
<td>8</td>
</tr>
<tr>
<td>Pharmaceutical Preparations</td>
<td></td>
</tr>
</tbody>
</table>

## 3. International Recommendations, Guidelines and other matters related to the manufacture and quality control of biologicals

### 3.1 Vaccines and related substances

<table>
<thead>
<tr>
<th>Subsection</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1.1 Guidelines for independent lot release of vaccines by regulatory</td>
<td>11</td>
</tr>
<tr>
<td>authorities</td>
<td></td>
</tr>
<tr>
<td>3.1.2 Recommendations for the evaluation of animal cell cultures as</td>
<td>11</td>
</tr>
<tr>
<td>substrates for the manufacture of biological medicinal products and for</td>
<td></td>
</tr>
<tr>
<td>the characterization of cell banks</td>
<td></td>
</tr>
<tr>
<td>3.1.3 Recommendations to assure the quality, safety and efficacy of</td>
<td>12</td>
</tr>
<tr>
<td>hepatitis B vaccines</td>
<td></td>
</tr>
<tr>
<td>3.1.4 Recommendations to assure the quality, safety and efficacy of live</td>
<td>13</td>
</tr>
<tr>
<td>attenuated yellow fever vaccines</td>
<td></td>
</tr>
<tr>
<td>3.1.5 Procedure for assessing the acceptability, in principle, of vaccines</td>
<td>14</td>
</tr>
<tr>
<td>for purchase by United Nations agencies</td>
<td></td>
</tr>
</tbody>
</table>

### 3.2 Blood products and related substances

<table>
<thead>
<tr>
<th>Subsection</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2.1 GMP for blood establishments</td>
<td>15</td>
</tr>
<tr>
<td>3.2.2 WHO tables on tissue infectivity distribution in TSEs</td>
<td>15</td>
</tr>
<tr>
<td>3.2.3 Assessment criteria for national blood regulatory systems</td>
<td>16</td>
</tr>
</tbody>
</table>

## 4. International reference materials – vaccines and related substances

### 4.1 WHO International Standards and Reference Reagents – vaccines and related substances

<table>
<thead>
<tr>
<th>Subsection</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1.1 Antibody to vaccinia virus</td>
<td>17</td>
</tr>
<tr>
<td>4.1.2 Antibody to pandemic H1N1 influenza virus</td>
<td>18</td>
</tr>
<tr>
<td>4.1.3 Tetanus vaccine</td>
<td>18</td>
</tr>
<tr>
<td>4.1.4 BCG vaccine</td>
<td>19</td>
</tr>
</tbody>
</table>

### 4.2 Proposed new projects – vaccines and related substances

<table>
<thead>
<tr>
<th>Subsection</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.2.1 Diphtheria vaccine for calibration of formulated products by</td>
<td>20</td>
</tr>
<tr>
<td>alternative to challenge assays (Vero cell assay)</td>
<td></td>
</tr>
</tbody>
</table>
4.2.2 Hepatitis B vaccine
4.2.3 BCG vaccine of Moreau substrain
4.3 Ongoing stability monitoring – vaccines and related substances
4.3.1 Tetanus toxoid, flocculation

5. International reference materials – blood products and related substances
5.1 WHO International Standards and Reference Reagents – blood products and related substances
5.1.1 Thrombosis and haemostasis
5.1.2 Antithrombin, plasma
5.1.3 Factors II, VII, IX and X, plasma
5.1.4 von Willebrand factor, concentrate
5.1.5 C1 inhibitor, plasma and C1 inhibitor, concentrate
5.2 Proposed new projects – blood products and related substances
5.2.1 Factor II, X, concentrate
5.2.2 Factor VII, concentrate
5.2.3 von Willebrand factor propeptide (VWFpp) as a new analyte to be added to the WHO Sixth International Standard for factor VIII/von Willebrand factor, plasma
5.2.4 High molecular weight urokinase
5.2.5 D-dimer, plasma
5.2.6 PAI-1 antigen, plasma
5.2.7 Transfusion-relevant bacterial strain panel

6. International reference materials – diagnostic reagents
6.1 WHO International Standards and Reference Reagents – diagnostic reagents
6.1.1 Human cytomegalovirus (HCMV) for nucleic acid amplification technique (NAT)-based assays
6.1.2 RHD/SRY plasma DNA
6.2 Proposed new projects – diagnostic reagents
6.2.1 Hepatitis B virus DNA for NAT-based assays
6.2.2 Hepatitis C virus RNA for NAT-based assays
6.2.3 Human herpes virus 6 for NAT-based assays
6.2.4 Adenovirus for NAT-based assays
6.2.5 Detection of *Mycoplasma*: NAT-based assays
6.2.6 *Babesia* antibody
6.2.7 Chikungunya virus RNA for NAT-based assays
6.2.8 Anti-HTLV I/II reference panel
6.2.9 *Plasmodium falciparum* antibody reference panel

7. International reference materials – biotherapeutics (other than blood products)
7.1 WHO International Standards and Reference Reagents – biotherapeutics (other than blood products)
7.1.1 Thyroid-stimulating antibody
7.1.2 Follicle-stimulating hormone, human, recombinant, for bioassay
7.1.3 Sex hormone-binding globulin
7.1.4 Granulocyte colony-stimulating factor
7.2 Proposed new projects – biotherapeutics (other than blood products) 37
  7.2.1 WHO reference panel anti-erythropoietin antibodies testing 37
  7.2.2 Human proinsulin 37
  7.2.3 Human insulin C-peptide 37
  7.2.4 Proposed WHO First Reference Reagent for human interleukin-29 37
  7.2.5 CD4+ cell counting standard 37
7.3 Ongoing stability monitoring – biotherapeutics (other than blood products) 37
  7.3.1 Somatropin (recombinant human growth hormone) 37

8. International reference materials – antibiotics 39
  8.1 WHO International Standards and Reference Reagents – antibiotics 39
    8.1.1 Vancomycin 39
  8.2 Proposed new projects – antibiotics 39
    8.2.1 Dihydrostreptomycin 39

References 40

Annex 1
  WHO Recommendations, Guidelines and other documents related to the manufacture and control of biological substances used in medicine 41

Annex 2
  Guidelines for independent lot release of vaccines by regulatory authorities 47

Annex 3
  Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks 79

Annex 4
  Recommendations to assure the quality, safety and efficacy of recombinant hepatitis B vaccines 189

Annex 5
  Recommendations to assure the quality, safety and efficacy of live attenuated yellow fever vaccines 241

Annex 6
  Procedure for assessing the acceptability, in principle, of vaccines for purchase by United Nations agencies 315

Annex 7
  Biological substances: International Standards and Reference Reagents 383
WHO Expert Committee on Biological Standardization
18 to 22 October 2010

Members

Dr M.M.F. Ahmed, National Organisation for Drug Control and Research (NODCAR), Agousa, Egypt
Dr R. Dobbelaer, Lokeren, Belgium
Dr J. Epstein, Center for Biologics Evaluation and Research, Food and Drug Administration, Rockville, MD, United States of America (USA)
Dr E. Griffiths, Health Canada, Ontario, Canada (Chairman)
Mrs T. Jivapaisarnpong, Division of Biological Products, Ministry of Public Health, Nonthaburi, Thailand (Vice-chairman)
Dr H. Klein, National Institutes of Health, Bethesda, MD, USA
Dr P. Minor, National Institute for Biological Standards and Control, Potters Bar, England
Dr L.S. Slamet, National Agency of Drug and Food Control, Jakarta, Indonesia
Dr P. Strengers, Sanquin, Amsterdam, the Netherlands
Professor H. Yin, State Food and Drug Administration, Beijing, China

Representatives from other organizations

Council of Europe, European Directorate for the Quality of Medicines and HealthCare
Dr K.H. Buchheit, OMCL Network and Health Care, Strasbourg, France
Mr J-M. Speiser, OMCL Network and Health Care, Strasbourg, France

Developing Country Vaccine Manufacturers’ Network
Dr M. Bhalgat, Biological E. Ltd, Hyderabad, India
Mr P.V.V.S. Murthy, Biological E. Ltd, Hyderabad, India

European Diagnostic Manufacturers Association
Dr C. Giroud, Bio-Rad Laboratories, Marnes-la-Coquette, France

European Medicines Agency
Dr P. Richardson, London, England

1 The decisions of the Committee were taken in closed session with only members of the Committee present. Each Committee member had completed a declaration of interests form prior to the meeting. These were assessed by the WHO Secretariat and no declared interests were considered to be a conflict for full participation in the meeting.

2 Unable to attend.
European Society of Human Genetics
Dr M. Morris, Centre médical universitaire, Geneva, Switzerland

International Association of Biologicals
Dr A. Mire-Sluis, Amgen Inc., Thousand Oaks, CA, USA

International Federation of Clinical Chemistry and Laboratory Medicine
Professor J-C. Forest, Québec, Canada

International Federation of Pharmaceutical Manufacturers & Associations
Dr M. Duchêne, GSK Biologicals, Wavre, Belgium
Dr A. Sabouraud, Sanofi Pasteur, Marcy l’Étoile, France
Dr C. Saillez, GlaxoSmithKline Biologicals, Wavre, Belgium

International Organization for Standardization
Mr T. Hancox, Geneva, Switzerland

International Plasma Fractionation Association
Dr R. Perry, Amsterdam, the Netherlands

International Society of Blood Transfusion
Professor E. Zhiburt, Moscow, Russian Federation

International Society on Thrombosis and Haemostasis
Professor K. Mertens, Sanquin, Amsterdam, the Netherlands

Plasma Protein Therapeutics Association
Dr I. von Hoegen, Brussels, Belgium

United States Pharmacopeia
Dr T.S. Morris, Rockville, MD, USA
Dr A. Szajek, Rockville, MD, USA

Secretariat
Dr J.W. Atkins, National Institutes of Health, Bethesda, MD, USA (Temporary Adviser)
Professor A. Barrett, University of Texas Medical Branch at Galveston, TX, USA (Temporary Adviser)
Professor D. Barton, Our Lady’s Children’s Hospital, Dublin, Ireland (Temporary Adviser)
Mr A. Battersby, Somerset, England (Temporary Adviser)
Dr V. Bogdanova, Federal Medico-Biological Agency (FMBA), Moscow, Russian Federation (Temporary Adviser)
Dr T. Burnouf, Human Protein Process Sciences, Lille, France (Temporary Adviser)
Professor K. Cichutek, Paul-Ehrlich-Institute, Langen, Germany (Temporary Adviser)
Dr M. Ferguson, Norfolk, England ( Temporary Adviser)
Dr P. Ganz, Biologics and Genetic Therapies Directorate, Health Canada, Ottawa, Canada (Temporary Adviser)

Mr A. Garnett, London, England (Temporary Adviser)

Ms L. Gomes Castanheira, Agencia Nacional da Vigilancia Sanitaria, Brasilia DF, Brazil (Temporary Adviser)

Dr A. Hubbard, National Institute for Biological Standards and Control, Potters Bar, England (Temporary Adviser)

Dr S. Inglis, National Institute for Biological Standards and Control, Potters Bar, England (Temporary Adviser)

Dr J. Joung, National Institute of Food and Drug Safety Evaluation, Korea Food and Drug Administration, Seoul, Republic of Korea (Temporary Adviser)

Dr C. Milne, OMCL Networks and HealthCare (DBO), European Directorate for the Quality of Medicines and HealthCare (EDQM), Strasbourg, France (Temporary Adviser)

Dr F. Moftah, Ministry of Health, Giza, Egypt (Temporary Adviser)

Dr S. Morgeaux, Agence Francaise de Sécurité Sanitaire des Produits de Santé (AFSSAPS), Lyons, France (Temporary Adviser)

Dr R. Murray, National Institutes of Health, Bethesda, MD, USA (Temporary Adviser)

Dr M. Nübling, Paul-Ehrlich-Institute, Langen, Germany (Temporary Adviser)

Professor D.E. Onions, CSO BioReliance, Almancil, Portugal (Temporary Adviser)

Dr H.J. Oh, Korea Food and Drug Administration, Seoul, Republic of Korea (Temporary Adviser)

Dr J. Petricciani, Palm Springs, CA, USA (Temporary Adviser)

Professor M. Pocchiari, Istituto Superiore di Sanita, Rome, Italy (Temporary Adviser)

Dr I. Sainte Marie, Agence Francaise de Sécurité Sanitaire des Produits de Santé (AFSSAPS), Saint Denis, France (Temporary Adviser)

Dr C. Schärer, Swissmedic, Bern, Switzerland (Temporary Adviser)

Professor R. Seitz, Paul-Ehrlich-Institute, Langen, Germany (Temporary Adviser)

Dr S. Shani, Central Drugs Standard Control Organisation, New Delhi, India (Temporary Adviser)

Dr R. Sheets, National Institutes of Health, National Institute of Allergy and Infectious Diseases (NIAID), Bethesda, MD, USA (Temporary Adviser)

Dr G. Smith, TGA, Canberra, Australia (Temporary Adviser)

Dr J. Southern, Ministry of Health, Cape Town, South Africa (Temporary Adviser)

Dr M. Valdkhani, Ministry of Health and Medical Education of the Islamic Republic of Iran, Tehran, Islamic Republic of Iran (Temporary Adviser)
Professor G.N. Vyas, University of California at San Francisco, San Francisco, CA, USA (Temporary Adviser)

Dr J.Z. Wang, National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China (Temporary Adviser)

Dr H. Watanabe, National Institute of Infectious Diseases (NIID), Tokyo, Japan (Temporary Adviser)

Dr D. Wood, World Health Organization, Geneva, Switzerland (Secretary)

Dr K. Zoon, Director, NIAID, National Institutes of Health, Bethesda, MD, USA (Temporary Adviser; Rapporteur)
Abbreviations

BCG  bacille Calmette–Guérin vaccine
BRN  Blood Regulators Network
BSP  Biological Standardisation Programme (EQDM)
CBER Center for Biologics Evaluation and Research
cfu  colony-forming units
CMV  cytomegalovirus
DTP  diphtheria–tetanus–pertussis vaccine
EDQM European Directorate for Quality of Medicines and Healthcare
ELISA enzyme-linked immunosorbent assay
FDA United States Food and Drug Administration
FSH  follicle-stimulating hormone
GCP  good clinical practice
G-CSF  granulocyte colony-stimulating factor
GCV  geometric coefficient of variation
GMP  good manufacturing practices
HBsAg  hepatitis B surface antigen
HBV  hepatitis B virus
HCMV  human cytomegalovirus
HCV  hepatitis C virus
HHV  human herpes virus
HI  haemagglutination–inhibition
HPLC  high-pressure liquid chromatography
HTLV  human T-cell lymphotropic virus
IL  interleukin
IPV  inactivated polio vaccine
ISBT  International Society of Blood Transfusion
ISTH  International Society on Thrombosis and Haemostasis
IU  International Unit
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVIG</td>
<td>intravenous immunoglobulin</td>
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<tr>
<td>NAT</td>
<td>nucleic acid amplification technique</td>
</tr>
<tr>
<td>NCL</td>
<td>national control laboratory</td>
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<tr>
<td>NIBSC</td>
<td>National Institute for Biological Standards and Control</td>
</tr>
<tr>
<td>NRA</td>
<td>national regulatory authority</td>
</tr>
<tr>
<td>OPV</td>
<td>oral polio vaccine</td>
</tr>
<tr>
<td>PAI</td>
<td>plasminogen activator inhibitor</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PEI</td>
<td>Paul-Ehrlich-Institute</td>
</tr>
<tr>
<td>PRNT</td>
<td>plaque reduction neutralization tests</td>
</tr>
<tr>
<td>PrP</td>
<td>prion protein</td>
</tr>
<tr>
<td>rhFSH</td>
<td>recombinant human follicle-stimulating hormone</td>
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<tr>
<td>SHBG</td>
<td>sex hormone-binding globulin</td>
</tr>
<tr>
<td>SSC</td>
<td>Scientific and Standardisation Subcommittee (ISTH)</td>
</tr>
<tr>
<td>TSAbs</td>
<td>thyroid-stimulating antibodies</td>
</tr>
<tr>
<td>TSEs</td>
<td>transmissible spongiform encephalopathies</td>
</tr>
<tr>
<td>TSH</td>
<td>thyroid-stimulating hormone</td>
</tr>
<tr>
<td>UNICEF</td>
<td>United Nations Children's Fund</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>VN</td>
<td>virus neutralization</td>
</tr>
<tr>
<td>WP-TTID</td>
<td>Transfusion-Transmitted Infectious Diseases Working Party</td>
</tr>
<tr>
<td>VWF</td>
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</tr>
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<td>von Willebrand factor propeptide</td>
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<td>World Health Assembly</td>
</tr>
<tr>
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<td>World Health Organization</td>
</tr>
</tbody>
</table>
1. Introduction

The World Health Organization (WHO) Expert Committee on Biological Standardization met in Geneva from 18 to 22 October 2010. The meeting was opened on behalf of the Director-General by Dr Jean-Marie Okwo-bele, Director, Immunization, Vaccines and Biologicals. Dr Okwo-bele emphasized that evidence-based international norms and standards for biological products, developed through rigorous, transparent, inclusive and authoritative processes, provided a solid foundation upon which health systems could build to deliver products of assured quality to address important public health problems. He noted that the Committee was responsible for establishing such norms and standards and that the development of proposals to be considered at the current meeting involved extensive preparations, in some cases over several years leading up to the meeting.

He pointed out that the decisions of the Committee would have important public health outcomes. For example, a revision to the vaccines prequalification procedure was proposed. Vaccine prequalification was a core activity of WHO, the primary purpose of which was to ensure that the vaccines purchased by United Nations procurement agencies would be consistently safe and effective under conditions of use for national immunization programmes. Approximately 64% of the total global infant population received prequalified vaccines. The prequalification process was described in a written procedure, which had last been updated in 2005. Since that time, the number and type of vaccines being offered for prequalification had become more diverse. Other challenges to the prequalification programme included the availability of new production technologies, multiple production sites and partnerships between manufacturers in developing and developed countries. The demand for prequalification evaluation had significantly increased and this trend was expected to continue and even to accelerate in the coming years. The proposed revised procedure was expected to be more efficient and more transparent. The immunization programmes in countries that were recipients of prequalified vaccines had voiced their expectation that quality would not be compromised in the revised process. Critical review from the Committee would be extremely important to meet these various expectations and to provide this assurance.

Dr Okwo-bele also drew attention to the WHO Guidelines on tissue infectivity distribution in transmissible spongiform encephalopathies (1), which would be considered at the current meeting. These guidelines had last been published in 2006. They remained valid, but new information on the distribution of tissue infectivity was now available. It was thus important, in the context of potential transmission of variant Creutzfeldt–Jakob disease through human blood and blood products, as well as through medicinal products prepared with bovine-derived materials, that an update be considered. The Committee would consider
a proposal in which some tissues were moved from the category of “tissues with no detectable infectivity” to the category of “lower-infectivity tissues”.

He noted that each of the documents considered by the Committee was intended to serve as a guide for national requirements. If a national regulatory authority (NRA) so desired, the guidelines could be adopted as definitive national requirements.

As usual, the Committee would consider a number of proposed new or replacement global measurement standards. These were central to the regulatory process, as they defined the International Unit that is used for dosing purposes for many biological medicines.

Dr Elwyn Griffiths was elected Chairman of the overall meeting, Mrs Teena Jivapaisarnpong as Vice-chairman and Dr Kathy Zoon as Rapporteur. The meeting was divided into parallel tracks. Dr Elwyn Griffiths was elected as Chairman, Mrs Teena Jivapaisarnpong as Vice-chairman and the WHO Secretariat as Rapporteur for the Vaccine and Biotherapeutics track, and Dr Harvey Klein as Chairman, Dr Paul Strengers as Vice-chairman and Dr Anthony Hubbard and Dr Micha Nübling as Rapporteurs for the Blood Products and Related Substances track. The Committee adopted the agenda (WHO/BS/10.2156) and the timetable proposed.
2. General

2.1 Current directions

2.1.1 Strategic directions in biological standardization

The Committee was informed of WHO priorities in biological standardization, and the importance of the lessons learnt from the H1N1 2009 influenza pandemic with respect to production and use of, and access to, vaccines for global public health emergencies. The Committee discussed the need to ensure the availability of influenza reagents and high-yielding seed lots in a timely manner and the use of new production systems and adjuvants for influenza vaccines.

New developments in global immunization were described, including the Decade of the Vaccine, which was a major new initiative for research, development and delivery of vaccines for the world’s poorest children. The Committee expressed its support for the new initiative and the opportunities it presented. The Committee supported the role of WHO in the development of standards to facilitate this initiative. It emphasized the importance of regulatory strengthening, especially in the countries where the production of vaccines was a new activity, so that only vaccines of assured quality were used in national immunization programmes.

The Committee was also informed of the World Health Assembly (WHA) resolution of 2010 (WHA 63.12) on the availability, safety and quality of blood products (2), which was based on related discussions at its previous meetings. Other topics presented to the Committee included a WHO initiative on genomics and public health; an international forum hosted by the American Association for Clinical Chemistry on clinical laboratory testing through harmonization; and WHO’s position on animal testing for product development and control.

The Committee noted and agreed with the importance of the described activities.

2.1.2 Vaccines and biological therapeutics: recent and planned activities in biological standardization

A review of WHO’s recent and planned activities relating to the standardization of vaccines and similar biotherapeutic products, was presented to the Committee. Included in the review was an update on current and potential future WHO collaborating centres that support the work of the Committee. WHO was encouraging its collaborating centres to be more involved in various product areas, and was making efforts to improve the administrative process for assignment and reassignment of such centres. The Committee commended these initiatives.

The Committee also reviewed WHO’s plans for development of written standards and updating of existing documents. New processes were described
for posting draft documents on the WHO web site for public comments and also for posting the documents recommended for adoption by the Committee, prior to their formal publication in the WHO Technical Report Series. The Committee welcomed the new processes and expressed its support for the priorities proposed for its meetings in 2011–2013 – namely dengue, bacille Calmette–Guérin (BCG) and diphtheria–tetanus–pertussis (DTP) vaccines, combined vaccines based on DTP, oral or injected polio vaccine, and malaria vaccine. The importance of continuing to develop standards in a proactive manner at an early stage of product development was emphasized.

The status of the WHO cell banks was presented and the Committee was informed that WHO Vero cell reference bank 10-87 had been shown to be free of porcine circoviruses and porcine parvoviruses.

The Committee expressed its support for the continuing work on similar biotherapeutics products. It pointed out that stem cells would be an area of development and growth over the next few years and recommended that WHO be involved in the area and be prepared to develop guidance as appropriate.

2.1.3 Blood products and related in vitro diagnostics: recent and planned activities in biological standardization

The Committee was presented with an overview of accomplishments and planned activities for blood products and related biologicals. Resolution WHA63.12 (2) was discussed; this addressed associated in vitro medical devices for the first time. The Committee noted that one intention of the resolution was to strengthen NRAs for regulatory oversight of blood and blood products. However, there was a need to identify financial partners and foster advocacy for this activity. The Committee supported the resolution and its implementation.

The WHO strategic plan for products derived from human and animal blood was discussed. The Committee was reminded of the recently established WHO Guidelines for the production, control and regulation of snake antivenom immunoglobulins (3). To complement the guidance document, a new WHO web site on the production and availability of snake antivenoms had been established. Maintaining the web site was a priority and the Committee supported its continuation. The key deliverables for 2010 included WHO Guidelines on good manufacturing practices (GMP) for blood establishments, updating of WHO tables on tissue infectivity distribution of transmissible spongiform encephalopathies (TSEs), assessment criteria for blood regulatory systems, and a workshop on blood products at the forthcoming International Conference of Drug Regulatory Authorities. The Committee agreed with these priorities.

The Committee was also informed of the “Achilles project”, which aimed to increase the availability of essential plasma-derived products for developing
countries, by supporting the implementation of national validated quality and safety standards for plasma for fractionation. Plans for the standardization of in vitro biological diagnostic technologies for the detection of microbial agents with an impact on the regulation and control of the safety of blood and blood products were also described. The Committee supported these activities.

2.2 Reports

2.2.1 WHO Blood Regulators Network

Dr Jay Epstein provided a report from the Blood Regulators Network (BRN), which had been established in response to the recommendations of the Committee in 2004. The membership is composed of representatives from NRAs that have a comprehensive responsibility for blood regulation, and the necessary expertise and capacity to address emerging public health challenges. He reviewed recent activities of the group, including examining the implications of identification of murine-leukaemia-virus-related human retroviruses; reviewing the draft document describing the development of assessment criteria for national blood regulatory systems; and technical support and advocacy for the resolution WHA63.12 (2). The group had exchanged information on a variety of issues, for example, maintaining the blood supply in the face of an influenza pandemic; a Q-fever outbreak in the Netherlands; and thrombotic adverse events associated with the use of Octagam (intravenous immunoglobulin 5%).

The Committee was informed that the agenda for the next BRN meeting included the process for establishing new memberships, completion of the assessment criteria for the national blood regulatory systems, review of the WHO storage and transport of time- and temperature-sensitive pharmaceutical preparations for relevance to blood components, and topical updates.

The Committee noted the report. It recommended that the terms of reference of the BRN be revised to clarify the process for admittance of new members.

2.2.2 Reports from the WHO international laboratories and collaborating centres for biological standards

The Committee was informed of recent developments at the WHO international laboratories and collaborating centres for biological standards.

National Institute for Biological Standards and Control (NIBSC), Potters Bar, England

The Committee was informed that NIBSC had a new influenza resource centre and that the stem cell bank in the United Kingdom had opened. A reorganization of the Health Protection Agency, which included NIBSC, was under way. This may have an impact on NIBSC’s role and responsibilities.
NIBSC had developed 14 of the proposed reference preparations for consideration by the Committee for adoption and establishment at the current meeting. In 2009, there had been a 31% increase in the number of WHO International Standards ordered by national control laboratories.

Some of the scientific highlights since the sixtieth meeting had included investigating the finding of porcine circoviruses in rotavirus vaccines; investigation of adverse events associated with intravenous immunoglobulin (IVIG); responding to H1N1 2009 pandemic influenza; and ongoing investigations of the contaminated heparin event. NIBSC had developed improved tests that had enabled anti-A and anti-B limits to be introduced for IVIG in one area of the world and thus reduce adverse events; had improved diphtheria potency testing; and had developed a rapid polymerase chain reaction (PCR)-based assay for cytokines. NIBSC was also opening up new areas, such as standards for genetic testing, standards for biosimilars and standards for the development of nucleic-acid-based diagnosis of infectious disease.

Commenting on the opening of the stem cell bank in the United Kingdom, the Committee noted that it was essential that development of cell-based medicines be underpinned by a sound regulatory science platform. It was noted that activities were under way in multiple countries, including Canada, the USA and countries in the European Union, to develop scientific principles and guidance in these areas. The Committee was supportive of this initiative and recommended sharing of information on the outcomes of these activities and on the safety of clinical trials using these products.

WHO Collaborating Centre for Quality Assurance of Blood Products and in vitro Diagnostic Devices, Paul-Ehrlich-Institute (PEI), Langen, Germany

PEI was redesignated in July 2009 as a WHO Collaborating Centre for Quality Assurance of Blood Products. The Institute was planning to expand the scope of its collaboration to include vaccines, gene therapy, and cell and tissue therapy. An overview of PEI’s organization and scope of responsibilities and of the collaborative workplan with WHO was presented. The workplan included initiatives such as the development of a panel of parvovirus B19 genotypes for nucleic acid amplification test (NAT)-based assays, development of an International Standard for hepatitis E virus RNA for NAT, and an International Standard for hepatitis C core antigen. PEI would also explore a new factor VIII potency assay. The Committee was informed that PEI had also developed training courses for regulatory authority personnel. PEI was working with other WHO collaborating centres on a variety of issues, including the BRN.

The Committee expressed its support for the collaborative efforts of PEI and commended the Institute’s efforts to provide training for NRA personnel from other countries.
Center for Biologics Evaluation and Research (CBER), United States
Food and Drug Administration (FDA), Bethesda, USA

The Committee was informed of the appointment of a new Director of CBER/FDA and a new Chief Scientist at FDA. Organizational changes at CBER were described, including a new Division of Product Quality, which would be involved in the quality management for the control and testing in support of product licensing and lot release, and maintenance and distribution of Reference Reagents. Lessons learnt from the H1N1 2009 pandemic were described, and ongoing initiatives in influenza preparedness, including new methods for selection of vaccine strains and reagent preparation, were presented. The projected availability of a replacement pneumococcal reference serum in 2011 was also described. New methods and reagents for testing poliovirus vaccines, developed at CBER, were described. An expanded role of CBER in global public health, particularly prevention, diagnosis and treatment of neglected tropical diseases, was an important new initiative. In this context, CBER had sponsored workshops on transmission-blocking malaria vaccines.

An overview of CBER activities related to blood and blood products was also presented, including the development of an International Standard for dengue virus types 1–4 RNA, the WHO First International Standard for antibodies to *Trypanosoma cruzi*, a human T-cell lymphotropic virus (HTLV) I and II antibody reference panel, and a malaria antibody reference panel. New proposals included work to evaluate a WHO First International Standard for Chikungunya virus RNA, a *Babesia microti* antibody reference panel, and WHO Reference Reagents for murine-leukaemia-related viruses.

The Committee recognized the importance of the international standardization activities of CBER.

2.3 Issues
2.3.1 Scientific issues identified by users of WHO biological reference preparations

The European Directorate for Quality of Medicines and Healthcare (EDQM) uses WHO biological reference preparations in its Biological Standardisation Programme (BSP). The goals of the BSP include establishment of Ph. Eur. Biological Reference Preparations; standardization of methods; application of the “3R concept” to refine, reduce or replace animal use; and international harmonization through collaboration with WHO and non-European partners.

The Committee was informed of a new test for rabies vaccine for veterinary use, which used serology instead of survival, following appropriate validation. It proposed that consideration be given to evaluating the new test for testing rabies vaccine for human use as well. The Committee agreed with the
concept of new “3R” assays to replace traditional animal survival tests, following appropriate validation. The Committee also agreed that these methods should be included in the WHO specifications for evaluation of products, when agreed upon, to encourage their wider uptake.

### 2.3.2 Issues shared with the WHO Expert Committee on Specifications for Pharmaceutical Preparations

Unusually, both the WHO Expert Committee on Biological Standardization and the WHO Expert Committee on Specifications for Pharmaceutical Preparations were meeting in parallel and so a joint session in which the two Committees met together had been organized to discuss issues of common interest. The session was chaired by Dr Griffiths and Dr Ivan Addae-Mensah, Chairman of the WHO Expert Committee on Specifications for Pharmaceutical Preparations.


The Committees reviewed a proposed WHO Guideline on regulatory oversight of storage and transport of time- and temperature-sensitive pharmaceutical products. The intent was to provide a comprehensive document covering regulatory expectations for the entire supply chain. The approach that had been taken in developing the document was to draw together advice from existing national guidance and standards and make this relevant to all countries. One of the goals of the Guideline was to harmonize existing guidance and standards. The document had also been designed to provide a logical structure to which “how to” documents could be linked in the future. After making suitable amendments, the Committees recommended that the Guideline be adopted and appended to the report of the WHO Expert Committee on Specifications for Pharmaceutical Preparations (4). In addition, the Expert Committee on Biological Standardization recommended that an addendum to the Guideline be developed for labile blood products. It was agreed by both Committees that the proposed “how to” documents should be developed to further explain how the appropriate regulatory oversight should be exercised.

**Proposed WHO First International Standard for biosynthetic human insulin (WHO/BS/10.2143 and QAS/10.381)**

The Committees considered a proposal to start a new project to establish a new International Standard for biosynthetic human insulin, with an assignment of content in mg/mg.

As background, the Committees were reminded that WHO currently distributed standards for the bioassay of therapeutic insulin, and for the
immunoassay of insulin in clinical diagnosis. Both were calibrated in International Units (IU), with no formally assigned content in mg. However, in therapeutic applications, insulin was now assayed in mg by high-pressure liquid chromatography (HPLC), and unitage was assigned using an internationally agreed conversion factor. In diagnostic immunoassays, the use of arbitrary units for small, well-characterized proteins, and value assignment by immunoassay, is no longer considered state of the art. To support these developments, it was proposed that WHO should either discontinue the existing standards, or establish an insulin standard value assigned in SI units (mg).

Currently, manufacturer, national and pharmacopoeial standards were established as primary standards, traceable to SI using a reference method. There was, however, no internationally agreed reference method. Theoretically, different organizations and agencies could assign the content of their standards using a variety of accepted methods, such as elemental nitrogen or mass balance. The uncertainty associated with the methods could lead to variability in assigned content and mean that data were neither comparable nor compatible.

The Committees were informed that requests had been received for an SI-traceable WHO standard for insulin from a consortium of stakeholders, including major insulin manufacturers in the USA and Europe, and at least one pharmacopoeia (representing the therapeutic community) and the American Diabetes Association (representing the diagnostic community). This proposal had been put to the Committee in 2009 in preliminary form, but at that time it was felt to require more extensive discussion. During 2010, the proposal had been widely circulated to stakeholders in both therapeutic and diagnostic communities, and a consultation had been held by teleconference. Although a majority appeared to be in favour of accepting the arguments and endorsing the proposal, this was not unanimous.

To resolve the impasse, it was now proposed to initiate a project to establish an insulin standard (with a labelled content in mg/mg), recognizing that all stakeholders now worked in SI units. This would not imply agreement or recommendation of its suitability for any particular application, which should be defined on the basis of further studies. For potential diagnostic application, it was proposed that studies should be conducted to address commutability issues; and that for potential therapeutic application, studies should be conducted to demonstrate a reduction in between-standard variability as a result of using such a standard. Agreement of stakeholders would also be needed for the principle of tracing local standards to a higher-order International Standard and it was proposed that the formal endorsement of any such application should be the subject of international agreement based on the results of these further studies.

The Committees agreed with the proposal to develop a reference preparation so that it continued to be possible to define the IU, and also to
investigate its potential use in the diagnostic area. The Committees considered that further work was needed to inform a decision on whether the proposed reference material would be of potential use in the therapeutic area. The Committees also requested WHO to publish a statement on the agreed conversion factor between IU and mg/mg.
3. International Recommendations, Guidelines and other matters related to the manufacture and quality control of biologicals

All Recommendations and Guidelines established in the meeting are listed in Annex 1, which provides an updated listing of all current WHO Recommendations and Guidelines related to the manufacture and quality control of biologicals.

3.1 Vaccines and related substances

3.1.1 Guidelines for independent lot release of vaccines by regulatory authorities

Vaccine lot release conducted by NRAs is part of the regulation of vaccines and involves the independent assessment of each individual lot of a licensed vaccine before it is released onto the market. This assessment is based, as a minimum, on the review of manufacturers’ summary protocols. It may be supplemented by other documents, such as the release certificate from another NRA/national control laboratory (NCL) and, in some circumstances, by testing that is independent of the manufacturers’ quality-control testing.

WHO provides support for lot release programmes through written and measurement standards, strengthening the lot release function of the NRAs and providing training. However, a need for further written guidance was identified at a WHO consultation held in Ottawa, Canada, in 2007. Early drafts of proposed new WHO guidelines had been considered by the Committee at its meetings in 2008 and 2009.

The Committee reviewed a further draft (WHO/BS/10.2128), which had incorporated its earlier recommendations and had benefited from further rounds of public consultation. After making suitable amendments, the Committee recommended that the Guidelines be adopted and appended to its report (Annex 2). The Committee also recommended that workshops on the implementation of the Guidelines be convened by WHO.

3.1.2 Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks

Cell substrates are cells used to manufacture biological products. It is well established that both cell substrates themselves and events linked to cell growth can affect the characteristics and safety of the resultant biological products. Therefore, a thorough understanding of the characteristics of the cell substrate is essential, in order to identify points of concern and to develop a quality-control system that addresses these points.
Mindful of recent advances in the use and quality control of new animal-cell substrates – particularly continuous cell lines and insect cells – the Committee concluded that an update to the WHO Requirements for the use of animal cells as in vitro substrates for the production of biologicals (5) should be prepared. In order to facilitate the resolution of regulatory and scientific issues related to the use of animal (including human) cell cultures as substrates for the production of biological products, WHO had initiated this revision of its requirements on cell substrates by establishing a study group.

The Committee considered a draft revision to WHO guidance (WHO/BS/10.2132), which had been prepared by the study group and was based on a wide range of consultations with individuals and organizations with expertise in this area. After making suitable amendments, the Committee recommended that the recommendations be adopted and appended to its report (Annex 3). The Committee also recommended that WHO should (a) urgently lead the development of a risk-assessment process for cell substrates during the post-marketing period; and (b) convene workshops on the implementation of the revised recommendations.

3.1.3 Recommendations to assure the quality, safety and efficacy of hepatitis B vaccines

Hepatitis B virus has an outer coat consisting of protein, lipid and carbohydrate, and bearing a unique antigen complex, the hepatitis B surface antigen (HBsAg). Virus recovered from the plasma of a hepatitis B carrier has been used to clone the HBsAg gene. The HBsAg gene has been inserted into yeast and mammalian cells by means of appropriate expression vectors. Antigen expressed in several species of yeast – namely *Saccharomyces cerevisiae*, *Pichia pastoris* and *Hansenula polymorpha* – and Chinese hamster ovary cells has been used to produce hepatitis B vaccines for more than 20 years.

The Committee was reminded that all hepatitis B vaccines currently on the market required formulation with adjuvants. Preservatives were used for multidose presentations but there were some single-dose presentations available without preservative. Recombinant hepatitis B vaccines were available as monovalent products or included in combination vaccines together with other antigens such as hepatitis A virus, diphtheria toxoid, tetanus toxoid, whole-cell oracellular pertussis components, *Haemophilus influenzae* type b conjugated antigen, and inactivated poliomyelitis viruses.

Following the development of hepatitis B vaccines containing HBsAg produced by recombinant DNA techniques in yeast, the first set of requirements was adopted by the Committee in 1986. These requirements were revised in 1988 to include vaccines produced by recombinant techniques in mammalian cells, as well as yeast cells. With the development and implementation of new in vitro
assays to determine antigen content, an amendment was published to include the use of the in vitro assay in the quality control of recombinant hepatitis B vaccines.

The Committee considered a proposed revision (WHO/BS/10.2130) that applied to vaccines containing HBsAg only and was intended to replace the WHO Requirements for hepatitis B vaccine made by recombinant DNA techniques (1989) (6), with a corresponding amendment in 1997 (7). After making suitable amendments, the Committee recommended that the Recommendations be adopted and appended to its report (Annex 4). In addition, the Committee recommended that (a) the suitability of the general safety test should be reviewed by WHO for all vaccines; and (b) a WHO International Standard reference preparation for non-adsorbed hepatitis B vaccine should be developed.

### 3.1.4 Recommendations to assure the quality, safety and efficacy of live attenuated yellow fever vaccines

Requirements for yellow fever vaccine were first formulated in 1958, by the WHO Study Group on Requirements for Yellow Fever Vaccine and Requirements for Cholera Vaccine (8). The Requirements embodied recommendations made by the WHO Expert Committee on Yellow Fever Vaccine at its first meeting (9), and they applied to vaccine prepared from a suitable strain of yellow fever virus. The vaccine was intended to be given by subcutaneous injection. Conformity with these Requirements was the basis for WHO approval of yellow fever vaccine used for vaccination and revaccination against yellow fever, in connection with certification for the purposes of international travel. Such approval was given only to vaccine prepared using seed derived from the 17D strain of yellow fever virus. Yellow fever continued to be the only disease for which a certificate of vaccination was required for entry into some countries, and the update of the International Health Regulations increased attention to the need for such certificates. The WHO Requirements were also used by NRAs for the control and approval of yellow fever vaccine used in national immunization programmes.

In 2008, the Expert Committee on Biological Standardization recommended that the Requirements for yellow fever vaccine (8) be reviewed, as it was more than 10 years since they had been updated, and sections on nonclinical and clinical evaluation for new candidate yellow fever vaccines were required. To facilitate this process, WHO convened a meeting of experts, regulatory professionals and other stakeholders, in Geneva in May 2009, to discuss the scientific basis for the present revision of the Requirements and to develop revised Recommendations for yellow fever vaccines.

At its current meeting, the Committee considered a draft update to the Recommendations (WHO/BS/10.2131). The scope of the proposed update encompassed live attenuated yellow fever vaccines derived from strain 17D, including 17D-204 and 17DD substrains. After making suitable amendments, the
Committee recommended that the Recommendations be adopted and appended to its report (Annex 5).

In addition, the Committee recommended that WHO give further consideration to: (a) the test in non-human primates that was specified in the document, including publication of available data from that test; (b) the impact of yellow fever vaccine titre on interference with co-administration of other vaccines; (c) the implications for other guidelines for live virus vaccines of the change in thermostability specifications (deletion of the need for a residual titre after heating); (d) development of a WHO International Standard for human serum antibodies; (e) renaming of WHO seed 213-77 as a WHO primary seed; and (f) use of the reference virus 168/73 as per Appendix 2 of the recommendations, and not as a potency reference.

3.1.5 Procedure for assessing the acceptability, in principle, of vaccines for purchase by United Nations agencies

WHO provides advice to the United Nations Children’s Fund (UNICEF) and other United Nations agencies on the acceptability, in principle, of vaccines considered for purchase by such agencies. This service is called prequalification. The purpose of the United Nations prequalification assessment is to provide assurance that candidate vaccines meet: (a) WHO recommendations on quality, safety and efficacy, including compliance with WHO’s recommended standards for GMP and good clinical practice (GCP); and (b) the operational packaging and presentation specifications of the relevant United Nations agency. The aim is to ensure that vaccines provided through the United Nations for use in national immunization services in different countries are safe, effective and suitable for the target populations at the recommended immunization schedules and with appropriate concomitant products.

The procedure in place at WHO to assess the acceptability of candidate vaccines for the United Nations was initially recommended for establishment by the Committee in 1988, and a number of published revisions to the procedure have been implemented (in 1996, 2002 and 2005).

The Committee considered a revision to the procedure (WHO/BS/10.2155) that addressed challenges faced by the vaccines prequalification programme – such as the increasing number of submissions and the increasing diversity and complexity of the products submitted to WHO for evaluation, as well as the ongoing maintenance of the prequalified status for those vaccines on the list of prequalified products. The latter included reassessments and reviews of variations, and investigation of quality and safety concerns reported by fieldworkers, which equated to a growing workload for WHO.

After making suitable amendments, the Committee recommended that the procedure be adopted and appended to its report (Annex 6). In addition, WHO was requested to consider: (a) adding a flow diagram of the procedure to
clarify the decision-making process, together with the timelines for each step; and (b) recording the opposition from the International Federation of Pharmaceutical Manufacturers and Associations to the concept of a “mandatory category” for programmatic suitability.

3.2 Blood products and related substances

3.2.1 GMP for blood establishments

The proposed WHO Guidelines on GMP for blood establishments (WHO/BS/10.2139 and QAS/10.382) aimed to provide guidance for both blood establishments and NRAs to establish, implement and enforce GMP in blood establishments. After initial discussion by the Committee in 2009, a draft of the Guidelines had been circulated for a wide global consultation during 2010, involving NRAs, blood establishments, national blood programmes and plasma fractionation associations in all WHO regions. The scope of the document included quality management, personnel, documentation, premises and equipment, qualification and validation, management of materials and reagents, manufacturing, contract manufacturing, analyses and services. Comments received during the consultation process strongly supported the need for this guidance document to facilitate enforcement and implementation of GMP for the manufacture of blood components in blood establishments and the availability of plasma for fractionation, as well as training programmes to strengthen the technical capacity of inspectorates in NRAs.

The Committee considered that implementation of the Guidelines would considerably improve the quality of blood collection on a global basis. It would also underpin the development and implementation of resolution WHA63.12 (2).

After making suitable amendments, the Committee recommended that the revised text be submitted to the Expert Committee on Specifications for Pharmaceutical Preparations, for review and adoption. This Expert Committee subsequently recommended that the Guidelines be appended to its report (10).

3.2.2 WHO tables on tissue infectivity distribution in TSEs

Since 2003, data on tissue infectivity distribution of TSEs in different species (humans, cattle, sheep and goats, elk and deer) have been continuously collected and assessed by a WHO-appointed expert group. The Committee was reminded that these data were of high importance for risk assessment of medicinal products manufactured with the use of biological materials. The Committee considered an update (WHO/BS/10.2152) to the 2006 revision of the tables (1). Tissue infectivity data in chronic wasting disease in elk and deer were included for the first time because of the ongoing spread of the disease and its potential transmission to other animal species.
Tissues were divided into high-infectivity tissues, lower-infectivity tissues and tissues without detectable infectivity or pathological prion protein (PrP<sup>TSE</sup>). Since 2006, some tissues (e.g. ovary, uterus) and body fluids (saliva, milk, urine) in which infectivity had not been previously detected had been found, by more sensitive methods, to contain infectivity or PrP<sup>TSE</sup>. Those had been moved from the category of “tissues with no detectable infectivity” to the category of “lower-infectivity tissues”. It was emphasized that categories of infectivity were not identical to categories of risk, since assessment of risk also considered the amount of tissue to which a person was exposed and the route of potential infection.

The Committee recommended that the proposed revised TSE infectivity tables be adopted and requested that the latest version of the tables be published on the WHO web site ([11]). The Committee additionally requested that the scope of application of these tables be expanded to include medical devices, in addition to pharmaceutical and biological products.

### 3.2.3 Assessment criteria for national blood regulatory systems

The Committee was informed of an initiative to develop assessment criteria, with the objective of promoting robust national blood regulatory systems through oversight by NRAs in both developed and developing countries. It was intended that the assessment tool would support either external or self-assessment exercises by NRAs for regulation of blood products and help identify areas for future development. The document focused on general areas such as the legal framework, plus essential functions, infrastructure and related quality elements. The list of essential functions in the tool was intended to represent best practice. The Committee noted, in this context, that not all functions might be carried out by the NRAs, and that all functions might not yet be fully in effect with the larger blood regulatory systems. The template was developed based on pre-existing WHO assessment templates for vaccines and medicinal products. Initial drafting of the tool had begun in 2008 and pilot self-assessments had been undertaken by Health Canada and Swissmedic in 2009–2010, to support preparation of the assessment criteria. Modifications had been introduced following this pilot phase and amendments had been agreed at the BRN meeting in October 2010.

The Committee noted that WHO intended to initiate an electronic consultation process with NRAs at different levels of regulation of blood products, to help identify the gaps and main priorities in developing countries. The draft tool would also be discussed in WHO training workshops to strengthen the technical assistance provided to NRAs.

The Committee supported the use of this normative tool to facilitate the introduction of regulations concerning blood products in all parts of the world, and to evaluate gaps or areas for improvement to sustain the development of resolution WHA63.12 ([2]), and requested that a final version be submitted for consideration at a future meeting.
4. International reference materials – vaccines and related substances

All reference materials established in the meeting are listed in Annex 7.

4.1 WHO International Standards and Reference Reagents – vaccines and related substances

4.1.1 Antibody to vaccinia virus

Standardization of anti-smallpox and anti-vaccinia titres in human serum or plasma has gained renewed interest following fears of the use of smallpox as a potential biological weapon and the possible need for mass vaccination. Prior to this resurgence of interest, standardization of anti-poxvirus titres had centred around the WHO First International Standard for anti-smallpox serum, which was established in 1966. This International Standard had been collected from convalescent smallpox patients but stocks had been destroyed after the discovery that the preparation was contaminated with HBV.

The Committee reviewed a proposal (WHO/BS/10.2134) that had the objective to establish the WHO First International Standard for anti-vaccinia plasma, and to establish traceability to the WHO First International Standard for anti-smallpox antibodies. A lyophilized candidate was prepared from a pool of defibrinated human plasma from vaccinees immunized with vaccinia virus. A collaborative study with nine laboratories from seven countries was performed. The characterization of the candidate preparation included the parallel testing of residual amounts of the WHO First International Standard for anti-smallpox antibodies, together with vaccinia immunoglobulin and anti-vaccinia samples. The obtained results were based on plaque reduction neutralization tests (PRNTs) and enzyme-linked immunosorbent assay (ELISA) methods. The study results showed variation for different PRNT assays, possibly associated with the origin of the virus (animal-derived versus cell culture-derived). Another source of variation was between the different methodologies used (PRNT versus ELISA). The potential approach to assign methodology-dependent unitages (as chosen for some national anti-vaccinia reference preparations) was discussed. Further analysis of the data obtained in the study, with a focus on sources of potential variation, was proposed by the Committee. There was no urgent need identified for the immediate establishment of this WHO International Standard, since well-characterized reference preparations from other sources were still available.

The Committee decided to postpone a decision regarding adoption of the preparation to a future meeting, with the expectation of a more detailed interpretation and analysis of the collaborative study data, including stratification by analytical methods and virus sources.
4.1.2 Antibody to pandemic H1N1 influenza virus

The Committee was reminded that haemagglutination–inhibition (HI) and virus neutralization (VN) serology assays were being used to assess the immunogenicity of many pandemic H1N1 (H1N1pdm) vaccines in clinical trials throughout the world, yet the assays were poorly standardized and previous studies had shown the results of such assays were variable between different laboratories.

The Committee considered a report (WHO/BS/10.2137) of a candidate reference material. A freeze-dried candidate preparation for H1N1pdm antibody had been prepared from pooled sera of subjects who had either recovered from recent pandemic influenza infection or had received an adjuvanted subunit pandemic H1N1 vaccine prepared from the reassortant virus NYMC X-179A (derived from influenza A/California/7/2009 (H1N1) virus). Ten laboratories from seven countries had tested the candidate preparation, coded 09/194, and a panel of human sera from convalescent subjects and recipients of influenza A/California/7/2009 (H1N1) pandemic vaccine; 10 had used HI assays and nine had used VN assays. A negative serum had also been included in the panel. The results demonstrated that the candidate WHO International Standard 09/194 would be of use in assays of antibody to influenza A/California/7/2009 (H1N1) virus vaccines.

The Committee agreed with the proposal that the preparation coded 09/194 be established as the WHO First International Standard for antibody to influenza A/California/7/2009 (H1N1) virus, with an assigned potency of 1300 IU per ampoule, i.e. 1300 IU/ml when reconstituted as directed with 0.5 ml of distilled water.

4.1.3 Tetanus vaccine

The Committee considered a proposal (WHO/BS/10.2150) to establish a preparation coded 08/218 as the WHO Fourth International Standard for tetanus vaccine.

The Committee was reminded that tetanus is caused by the action of a highly potent neurotoxin produced by the anaerobic bacterium, Clostridium tetani. The disease usually occurs through infection of a penetrating injury with tetanus spores, and neonatal tetanus occurs through infection of the umbilicus when the cord is cut with an unclean instrument, or when substances contaminated with tetanus spores are applied to the umbilicus stump. Immunity to tetanus is induced only by vaccination in childhood, and protective immunity is maintained by periodic revaccination throughout adulthood.

The supply of effective vaccines is dependent, among other factors, on confirmation of vaccine potency. The potency specifications for tetanus vaccines are dependent on the use of the WHO International Standard or material
The WHO Third International Standard for tetanus toxoid, adsorbed (coded 98/552) was established by the Committee in 2001. This material, with an assigned unitage of 469 IU/ampoule (from guinea-pig challenge assays), was used extensively as a working reference preparation and for calibration of in-house reference preparations according to the intention of WHO. The demand for this standard preparation had increased significantly in the past decade, as a result of the introduction of new combination vaccines containing tetanus toxoid. Owing to a rapid fall in available stocks at NIBSC, and on request by WHO, a project had been initiated to calibrate and establish a replacement standard calibrated against the existing standard, so that continuation of IU and traceability can be maintained among vaccine manufacturers and NRAs.

In the collaborative study reviewed by the Committee, 34 laboratories in 22 countries (Argentina, Australia, Belgium, Canada, China, Croatia, Cuba, France, Germany, Hungary, India, Indonesia, Italy, Japan, Mexico, the Netherlands, Norway, the Republic of Korea, the Russian Federation, South Africa, the United Kingdom and Viet Nam) participated. Six of these performed guinea-pig protection assays for calibration of the replacement WHO standard and 24 performed challenge assays in mice.

The Committee endorsed the proposal to establish the preparation 08/218 as the WHO Fourth International Standard for tetanus vaccine, with an assigned potency of 490 IU/ampoule, assigned on the basis of potency determination in guinea-pig assays, and noted that this proposal ensured continuity of unitage back to the WHO First International Standard.

The Committee agreed with a proposal that users wishing to use the mouse model for monitoring consistency of product could do so. The Committee further noted and endorsed the proposal to establish a working group to review and make recommendations on the relationship between the clinical efficacy and potency measured in mice, and to monitor the impact of using the mouse assay to determine the potency of tetanus vaccines. Data from EDQM supporting the use of the proposed WHO Fourth International Standard in the mouse model, with the proposed unitage of 260 IU/ml, indicating suitability for the assay of vaccines, were noted by the Committee, and it was requested that a complete report be submitted to WHO when it became available.

4.1.4 BCG vaccine

The Committee had previously established two BCG substrain preparations (Danish 1331 and Tokyo 172-1) as WHO Reference Reagents. However, owing to the absence of moisture content data for a third proposed candidate preparation,
BCG vaccine, Russian BCG-I substrain, the Committee agreed to defer establishment of the Russian BCG-I substrain preparation and requested that “the moisture content for the Russia BCG-I reference material be provided and reviewed and found acceptable by the Committee”.

The Committee reviewed the additional moisture content and real-time stability data (also in comparison with the other two established WHO Reference Reagents for BCG vaccines) on the Russian BCG-I substrain preparation (WHO/BS/10.2148).

The Committee endorsed the proposal to establish the preparation 07/274 as the WHO First Reference Reagent for BCG vaccine, Russian BCG-I substrain, and assigned 3.39 million colony-forming units (cfu)/ampoule and 7.5 ng of ATP per ampoule to it. It noted that the preparation was not intended to be used as a calibration standard and the ampoule contents were intended for guidance for users.

4.2 Proposed new projects – vaccines and related substances

4.2.1 Diphtheria vaccine for calibration of formulated products by alternative to challenge assays (Vero cell assay)

The Committee endorsed a proposed new project (WHO/BS/10.2153) to investigate the suitability of the WHO Fourth International Standard for diphtheria vaccine, to serve as a standard for Vero cell serology assays.

4.2.2 Hepatitis B vaccine

A standardized universal in vitro assay for the potency/antigen content of recombinant hepatitis B vaccines would be used to monitor batch-to-batch consistency. The Committee endorsed a proposed new project (WHO/BS/10.2153) on the development of an in vitro assay for hepatitis B vaccine for this purpose.

4.2.3 BCG vaccine of Moreau substrain

The Committee endorsed a proposed new project (WHO/BS/10.2153) on the development of a Reference Reagent for BCG vaccine of Moreau substrain, with the characterization to include both viable count assays and ATP assays, and also substrain identification using molecular biology methods.

4.3 Ongoing stability monitoring – vaccines and related substances

4.3.1 Tetanus toxoid, flocculation

Data from evaluation of the WHO International Standard for tetanus toxoid, for use in flocculation tests, preparation coded 04/150, were reported to the Committee (WHO/BS/10.2136). Samples tested by flocculation test after 4 years’
storage at various temperatures were combined with previous data. Relative activities were used to fit an Arrhenius equation relating degradation rate to absolute temperatures, and this was used to predict degradation rates when stored at –20 °C. The predicted loss of activity per year when stored at –20 °C was calculated as 0.032%.

The Committee endorsed the proposal that the preparation could now be considered to be stable until replacement, or 20 years after filling, whichever was sooner.
5. International reference materials – blood products and related substances

5.1 WHO International Standards and Reference Reagents – blood products and related substances

5.1.1 Thrombosis and haemostasis

The Committee was informed of the role of the Scientific and Standardisation Subcommittee of the International Society on Thrombosis and Haemostasis (SSC/ISTH) in the expert review of proposed WHO International Standards in the area of haemostasis. This activity was linked to WHO through the WHO-ISTH Liaison Group. The Committee was presented with the list of proposed WHO International Standards in the area of haemostasis that had been endorsed by the SSC/ISTH since the previous meeting of the Committee. Five proposed standards were reviewed, as follows: the WHO Third International Standard for antithrombin, plasma; the WHO Fourth International Standard for factors II, VII, IX, X, plasma; the WHO Second International Standard for von Willebrand factor (VWF), concentrate; the WHO First International Standard for C1 inhibitor, plasma; and the WHO First International Standard for C1 inhibitor, concentrate.

5.1.2 Antithrombin, plasma

The WHO Third International Standard for antithrombin, plasma is used to calibrate reagents used for the diagnosis of antithrombin deficiency. The Committee considered a report (WHO/BS/10.2146) in which a pooled normal plasma candidate was value assigned for function and antigen, in a collaborative study involving 24 laboratories from 13 countries. Estimates calculated relative to the WHO Second International Standard were associated with low interlaboratory variability for both function (geometric coefficient of variation (GCV) 2.5%) and antigen (GCV 4.4%). Estimates for antigen calculated relative to the local pools were not significantly different from estimates from the WHO Second International Standard. Estimates for function calculated relative to the local pools were significantly different from estimates calculated using the WHO Second International Standard but the mean values differed by only 2%. In the interests of continuity of the IU, it was proposed that mean values calculated relative to the WHO Second International Standard be assigned to the proposed WHO Third International Standard.

The Committee recommended the adoption of the preparation coded 08/258 as the WHO Third International Standard for antithrombin, plasma, with the following assigned values: function 0.95 IU/ampoule; antigen 0.96 IU/ampoule.
5.1.3 **Factors II, VII, IX and X, plasma**

The Committee was reminded that the existing reference preparation was used for the potency estimation of four factors in plasma. A pooled normal plasma candidate underwent value assignment in a collaborative study involving 29 laboratories in 14 countries (WHO/BS/10.2145). The majority of laboratories used clotting assays but a small number of chromogenic assays were also included. The interlaboratory variability of all four factors was extremely low for estimates calculated relative to the WHO Third International Standard, with GCVs below 3%. Mean estimates for factors VII, IX and X calculated relative to the local normal pools, were 5–6% lower than estimates relative to the WHO Third International Standard. This was probably caused by variability of local pools between collaborative studies. The excellent stability of the WHO Third International Standard supported the proposal to assign values calculated relative to the WHO Third International Standard and this route offered the best continuity of the IU for the proposed WHO Fourth International Standard.

The Committee adopted the preparation coded 09/172 as the WHO Fourth International Standard for factors II, VII, IX and, X, plasma, with the following assigned potencies:

- factor II 0.89 IU/ampoule
- factor VII 0.99 IU/ampoule
- factor IX 0.86 IU/ampoule
- factor X 0.89 IU/ampoule.

5.1.4 **von Willebrand factor, concentrate**

The existing WHO International Standard for von Willebrand factor is used for the potency labelling of VWF concentrates applied to replacement therapy for the bleeding disorder von Willebrand disease. Declining stocks have made it necessary to prepare a replacement standard. Three candidate concentrates underwent testing for three analytes (VWF:Ag, VWF:RCo and VWF:CB) relative to the current WHO First International Standard for von Willebrand factor, concentrate and the WHO Sixth International Standard for factor VIII/von Willebrand factor, plasma, in a collaborative study involving 45 laboratories from 12 different countries (WHO/BS/10.2147). One candidate was associated with the lowest interlaboratory variability for all three analytes and resembled the current WHO First International Standard for von Willebrand factor, concentrate, most closely, in terms of multimer profile and the ratio of VWF:antigen to VWF:RCo. Therefore, candidate D offered the best continuity for the IU between the WHO First and Second International Standards. The interlaboratory variability for
VWF:CB estimates was sufficiently low (GCV 19%) to allow the calculation of a valid consensus mean and enable first introduction of an IU standard for VWF:CB. There was also no significant difference between results obtained using different collagen reagents.

It was proposed that the concentrate coded 09/182 be accepted as the WHO Second International Standard for von Willebrand factor, concentrate, with the following assigned values:

- VWF:Ag 10.7 IU/ampoule
- VWF:RCo 9.2 IU/ampoule
- VWF:CB 10.3 IU/ampoule.

The Committee approved the adoption of 09/182 as the WHO Second International Standard for von Willebrand factor, concentrate, with the proposed assigned values.

5.1.5 **C1 inhibitor, plasma and C1 inhibitor, concentrate**

Deficiency of C1 inhibitor is associated with hereditary angioedema, which is treated by replacement therapy using purified concentrates of C1 inhibitor. The Committee considered a report (WHO/BS/10.2144) in which two new standards were proposed, a plasma standard for diagnosis of deficiency and a concentrate standard for potency labelling of purified concentrates. Value assignment involved a collaborative study with 28 laboratories from 13 countries. Both candidates were assayed for C1 inhibitor function relative to local plasma pools, in which it was proposed that 1 IU is defined as the amount of C1 inhibitor in 1 ml of pooled normal plasma. Estimates for the proposed WHO International Standard for plasma were associated with good agreement between laboratories (GCV 9.9%) and no difference between methods. Estimates for the proposed International Standard for concentrate showed a marked difference between the two methods, which indicates that a plasma standard is not suitable for the assay of purified concentrates. The concentrate standard had been evaluated by manufacturers and control laboratories and found satisfactory for the assay of different therapeutics.

The Committee recommended that the preparation coded 08/262 be adopted as the WHO First International Standard for C1 inhibitor, plasma, with an assigned value of 0.89 IU per ampoule and the preparation coded 08/256 as the WHO First International Standard for C1 inhibitor, concentrate, with an assigned value of 9.6 IU per ampoule. The Committee noted that the new concentrate standard had not been evaluated for the potency estimation of recombinant products and requested that this be reflected in the “instructions for use”.
5.2 Proposed new projects – blood products and related substances

5.2.1 Factor II, X, concentrate
The Committee considered a proposal (WHO/BS/10.2153) to replace the WHO Third International Standard for factor II, X concentrate, stocks of which were predicted to expire approximately 2 years after the current meeting and a replacement was required. This standard was used for the potency estimation of factor II and X in prothrombin complex concentrates and monocomponent factor IX concentrates. The Committee commented that sourcing of candidate materials could be problematic, since they should be obtained prior to formulation with antithrombin or heparin.

The Committee endorsed the proposed project.

5.2.2 Factor VII, concentrate
The Committee was informed that stocks of the WHO First International Standard for factor VII, concentrate would probably be exhausted approximately 2 years after the current meeting and a replacement was required (WHO/BS/10.2153). This standard was used for the potency estimation of factor VII in therapeutic concentrates. Candidate replacement preparations would consist of monocomponent factor VII therapeutic concentrates and would be value assigned using clotting and chromogenic methods.

The Committee endorsed the proposed project.

5.2.3 von Willebrand factor propeptide (VWFpp) as a new analyte to be added to the WHO Sixth International Standard for factor VIII/von Willebrand factor, plasma
Estimation of VWFpp is a new diagnostic approach to detect a subtype of von Willebrand disease associated with a decreased plasma half-life of VWF. The Committee considered a proposal (WHO/BS/10.2153) to assign a value for VWFpp as an addition to the current WHO Sixth International Standard for factor VIII/von Willebrand factor, plasma.

The Committee endorsed the proposed project.

5.2.4 High molecular weight urokinase
The Committee was informed that the WHO International Standard for urokinase used for the potency estimation of the thrombolytic drug and stocks of the WHO First International Standard would probably be exhausted approximately 2 years after the current meeting and a replacement was required (WHO/BS/10.2153). They were advised that the small number of manufacturers and laboratories measuring urokinase may limit the size of the calibration exercise for a WHO
Second International Standard. The possibility of dual labelling (IU and moles) of the replacement reference preparation was proposed.

The Committee endorsed the proposed project.

5.2.5 **D-dimer, plasma**

D-dimer is a fibrin breakdown product in plasma and is used widely for the diagnosis of thrombotic conditions such as deep vein thrombosis and pulmonary embolism. The Committee was informed that there are many different commercial methods and standardization between methods is very poor. A proposed new WHO International Standard (WHO/BS/10.2153) could be used by manufacturers to calibrate internal standards, so improving harmonization of measurement. The Committee noted that collection of source material may be problematic and would probably involve pooling plasma from patients with high D-dimer levels.

The Committee endorsed the proposed project.

5.2.6 **PAI-1 antigen, plasma**

Estimation of plasminogen activator inhibitor-1 (PAI-1) antigen in plasma has relevance as a marker for cardiovascular disease and diagnosis of metabolic syndrome and as a prognostic indicator for some cancers. A collaborative study on five plasma samples with different levels of PAI-1 antigen had indicated large variability between methods; however, this was reduced if results were calculated relative to preparations with consensus mean values. The Committee considered a proposal to assign a valid gravimetric value (ng) to one of these samples and to propose this as a new WHO International Standard (WHO/BS/10.2153).

The Committee endorsed the proposed project.

5.2.7 **Transfusion-relevant bacterial strain panel**

Bacterial contamination of platelet concentrates is a significant problem in transfusion medicine. To address this challenge of blood safety, both screening and pathogen-inactivation systems had been developed. These systems needed to be evaluated with appropriate materials. A four-member panel consisting of deep-frozen strains of different bacterial species (*Staphylococcus epidermidis, Klebsiella pneumoniae, Streptococcus pyogenes* and *Escherichia coli*) able to grow in platelet concentrates after spiking with low bacterial counts had been characterized in an international collaborative study. This study was performed in association with the Transfusion-Transmitted Infectious Diseases Working Party (WP-TTID) of the International Society of Blood Transfusion (ISBT). It revealed consistent results concerning definition of bacterial count, suitability for low-dose spiking and donor-independent growth properties in platelet concentrates collected in different regions of the world.
The Committee had previously requested stability data for the deep-frozen suspensions of bacteria. The Committee considered stability data for the four-member panel (WHO/BS/10.2154) and agreed that the preparations showed sufficient stability. The Committee endorsed the above four bacterial strains as a repository for transfusion-relevant bacterial strains. The Committee requested that detailed instructions for use, indicating the intended use of these materials and their storage, be prepared.

The Committee also considered additional information (WHO/BS/10.2154) on whether to recommend the expansion of the panel by addition of further platelet-relevant bacterial strains. Expansion of the project with a further nine platelet concentrate-growing strains representing different bacterial species (*Bacillus cereus* (spores), *Staphylococcus aureus*, *Streptococcus agalactiae*, *Enterobacter cloacae*, *Morganella morganii*, *Proteus mirabilis*, *Pseudomonas fluorescens*, *Salmonella choleraesuis* and *Serratia marcescens*) was proposed, after consultation with the ISBT WP-TTID Subgroup Bacteria, and further stakeholders.

The Committee noted the need for consideration of slow-growing bacterial species (e.g. Mycobacteria) to be added to the panel, and for the current restriction on use of the panel to investigate or validate platelet concentrates-associated screening. Bacterial strains suitable for validation of safety techniques developed for red cell concentrates may be considered in the future, as well as panels suitable for validation of pathogen-inactivation technologies. Furthermore, the need for ready-to-use materials (without a propagation step at the user’s laboratory) was identified. The Committee noted that tests had not been done on whether growth was obtained reproducibly after a frozen preparation had been diluted and directly inoculated into the cell preparations. If reproducible growth did not occur, then the strains would have to be lyophilized. A rationale for not testing lyophilized specimens, if this was not done, should be included in future reports.

The Committee endorsed the proposal for addition of the nine further bacterial strains suitable for control of contamination of platelet concentrates within the same repository. Appropriate characterization would need to be performed in an international collaborative study, following WHO recommendations, and submitted to the Committee, before adoption by WHO.

It was noted that this repository would be held and distributed by PEI, subject to the formal commitment of the institute for the distribution of WHO biological reference preparations.
6. International reference materials – diagnostic reagents

6.1 WHO International Standards and Reference Reagents – diagnostic reagents

6.1.1 Human cytomegalovirus (HCMV) for nucleic acid amplification technique (NAT)-based assays

The Committee reviewed a project (WHO/BS/10.2138) to evaluate the suitability of a proposed WHO First International Standard for HCMV.

HCMV is a ubiquitous herpesvirus with a high seroprevalence worldwide. It causes disease in immunologically naive individuals, such as neonates and infants, and immunosuppressed individuals, particularly transplant recipients and AIDS patients. Severe and life-threatening HCMV infections in immunocompromised individuals are managed through the administration of anti-herpetic agents; however, all are associated with toxicity with prolonged use.

The clinical utility of viral load measurements in the diagnosis and antiviral management of HCMV in transplant recipients has been well documented. Two therapeutic approaches have evolved – prophylaxis, whereby antiviral drugs are administered for a fixed period from the time of transplant, and pre-emptive treatment, which is administered in response to an increased risk of CMV disease. The pre-emptive approach requires diagnosis of HCMV replication, and initiation of antiviral therapy when a predetermined level of virus in peripheral blood is reached, prior to the appearance of clinical symptoms. Subsequently, the levels of virus are frequently measured in order to monitor the response to, and determine the duration of, treatment. Although there is no consensus on the optimal sample type or frequency of testing, both plasma and whole blood provide prognostic information.

NAT diagnostics for HCMV still lack standardization, which negatively impacts both patient management and comparison of clinical practice. Four HCMV preparations (strain Merlin lyophilized, strain Merlin liquid frozen, strain AD 169 lyophilized and purified BAC-cloned Merlin-DNA) were included in a collaborative study. Participants were requested to use their usual patient matrix (e.g. serum, plasma, whole blood) for the dilution series. The study involved 32 laboratories from 14 countries, which evaluated the candidate preparations using their routine HCMV NAT assays (53 quantitative, 5 qualitative NAT assays). Interlaboratory agreement for estimates of test virus samples was clearly improved when potencies were expressed relative to the candidate WHO International Standard (strain Merlin, lyophilized). Unexpectedly, there was no improvement in interlaboratory agreement for estimates in relation to the purified plasmid CMV-DNA sample and, consequently, no improvement of standardization between assays would be achieved by choosing this sample as a common reference.
Though commutability was not the main focus in this collaborative study, commutability between the different specimens containing the whole virus (like patient samples) was considered sufficiently confirmed. More rigorous determinations of commutability would raise issues applicable to the establishment of many other WHO International Standards and reference materials. The Committee noted that the use of the patient sample matrix to exclude or minimize matrix effects was relevant to assess commutability. In particular, it was noted that only one laboratory in the collaborative study had studied detection of HCMV in urine, suggesting that a further study of commutability in this matrix might be important.

The Committee adopted the preparation coded 09/162 as the WHO First International Standard for human cytomegalovirus for NAT-based assays, with an assigned potency of $5 \times 10^6$ IU per ml.

6.1.2 **RHD/SRY plasma DNA**

Determination of fetal RHD genotype using fetal DNA from the maternal circulation is increasingly used to manage pregnancies at risk for haemolytic disease of the newborn caused by maternal anti-D. The Committee considered a collaborative study involving 19 laboratories in 17 countries (WHO/BS/10.2149) that assessed the suitability of a freeze-dried plasma preparation, consisting of RhD-positive male plasma diluted in RhD-negative female plasma, as a WHO Reference Reagent (minimum sensitivity standard) for the detection of RHD and SRY genes using PCR. Overall, six different targets were used to detect the RHD gene and three different targets were used to detect DNA sequences on the Y chromosome. It was concluded that a dilution of 1 in 2 would be a suitable minimum dilution at which both RHD and SRY should be detected. It was noted that data on the comparative sensitivity of DNA detection of RHD compared with methods that are dependent on amniocentesis were not available. A concern was also raised with the use of a single male donor to prepare the candidate preparation and the possibility that this may impair the detection of genetic variants of RHD. However, it was accepted that this was unlikely since most laboratories use more than one target to detect the RHD gene.

The Committee approved the adoption of 07/222 as the WHO First Reference Reagent for RHD/SRY plasma DNA sensitivity. No units were assigned to this preparation.

6.2 **Proposed new projects – diagnostic reagents**

6.2.1 **Hepatitis B virus DNA for NAT-based assays**

Hepatitis B virus (HBV) DNA for NAT-based assays is essential for the safety testing of blood donations, cells and tissues and in the management of HBV infection and diagnosis of disease. The Committee was informed that stocks of
the current WHO Second International Standard for HBV DNA for NAT-based assays would probably be exhausted within 2–3 years after the meeting and a replacement was required (WHO/BS/10.2153). The WHO First and Second International Standards for HBV DNA for NAT-based assays were prepared from dilutions of the Eurohep R1 reference material (genotype A, HBsAg subtype adw2) in pooled human plasma. The proposal was to prepare the WHO Third International Standard from the same Eurohep R1 material, which was still present at NIBSC, and following the previous protocols. The evaluation of the candidate WHO Third International Standard for HBV DNA for NAT-based assays would be performed against the WHO Second International Standard. The Committee noted a suggestion to use pooled human plasma that has been tested negative for anti-HBs, as dilution matrix.

The Committee endorsed the proposed project.

6.2.2 Hepatitis C virus RNA for NAT-based assays

The Committee was informed that stocks of the current WHO Third International Standard for hepatitis C virus (HCV) RNA for NAT-based assays would probably be exhausted within 2–3 years after the meeting and a replacement was required (WHO/BS/10.2153). The Committee understood that the WHO International Standard for HCV RNA was essential for the calibration of secondary reference materials and the validation of HCV NAT-based assays used in the safety testing of blood and blood products. The WHO Third International Standard for HCV RNA for NAT-based assays (06/100) comprised genotype 1a. In parallel to the WHO Third International Standard for HCV (06/100), another batch of the same material was freeze-dried (06/102). Both batches were already characterized in the collaborative study for the WHO Third International Standard for HCV RNA for NAT-based assays and revealed mean relative potencies of 5.19 and 5.41 log_{10} IU/ml. It was proposed to perform a small study for comparison of 06/100 and 06/102 and to evaluate the stability. Based on the study outcome, establishment of the replacement WHO International Standard would be proposed at a future Committee meeting.

The Committee endorsed the proposed project.

6.2.3 Human herpes virus 6 for NAT-based assays

Human herpes virus 6 (HHV-6) is a ubiquitous virus with a high worldwide seroprevalence (>95%). HHV-6 variants have been identified, with variant B causally linked with diseases (e.g. febrile illness in children <2 years). Chromosomal integration of the HHV6 genome is detectable in 1–2% of individuals, which may be associated with high-level viraemia. Reactivation of latent infection in immunocompromised patients, e.g. transplant recipients, may result in severe disease. However, (quantitative) NAT is widely recommended
for the diagnosis of active HHV-6 infection and disease in children and immunocompromised individuals and to monitor the response to antiviral therapy. Diverse quantitative NAT systems have been mainly developed in-house without common standardization.

The Committee considered a proposal to initiate a project to evaluate cell-grown HHV-6 variant B as virus source and to characterize the material as a potential WHO International Standard, in a collaborative study (WHO/BS/10.2153). The target concentration for the preparation would be $10^7$ viral genomes/ml. Anticipated uses of such a standard would be in the validation and calibration of NAT-based assays for HHV-6 and to calibrate secondary reference standards. Patient samples would be included in the collaborative study, to assess commutability.

The Committee endorsed the proposed project.

6.2.4 Adenovirus for NAT-based assays

Adenovirus is a common human infection, with at least 52 serotypes and 7 subgroups. A wide spectrum of diseases is associated with this virus group (e.g. respiratory and gastrointestinal diseases, cystitis and conjunctivitis) in immunocompromised individuals, particularly in paediatric haematopoietic stem cell transplantation. The infection is associated with significant mortality and morbidity. In many countries, diagnosis and management of adenovirus infections relies on quantitative NATs, in most cases of generic design. A wide range of in-house and commercial NAT-based assays are in use, and there is a need to standardize them.

The Committee considered a proposal (WHO/BS/10.2153) to calibrate a candidate material for establishment as a WHO First International Standard for adenovirus for NAT-based assays, in an international collaborative study. The source virus would represent subgroup C subtype 2 (characterized by worldwide prevalence). It would be whole virus, grown in cell culture, with a target concentration of $10^7$–$10^8$ genomes/ml. While it was recognized that a single adenovirus strain might not represent all virus types, the selection of adenovirus subgroup C, subtype 2 was considered appropriate.

The proposed WHO International Standard would be of use for the validation and calibration of NAT-based assays and to calibrate secondary reference standards. The Committee recommended that the study include adequate representative assays in both urine and blood.

The Committee endorsed the proposed project.

6.2.5 Detection of Mycoplasma: NAT-based assays

The monophyletic bacterial class Mollicutes (trivial name: Mycoplasmas) is characterized by several unusual features (e.g. absence of a cell wall, small
Mollicutes are contaminants of biological processes, e.g. cell cultures, leading to changes in cell metabolism and/or phenotype. Various regulations for biological medicines therefore require testing of manufacturing components for the absence of Mollicutes, with culture-based methods now increasingly being replaced by NAT-based assays. However, there is a lack of standardization between different NAT techniques.

The Committee considered a proposal (WHO/BS/10.2153) to evaluate a candidate reference preparation as a potential WHO First International Standard for Mycoplasma for NAT-based assays. A pilot study would be performed to finally select the candidate material and to study the effect of lyophilization. In this feasibility study, the candidate species would be a frequent contaminant, Acholeplasma laidlawii, which would be tested in comparison to other relevant Mycoplasma spp. to investigate commutability. After selection of the candidate material, a global collaborative study would be performed to characterize the candidate WHO First International Standard for Mycoplasma, which was expected to be of use for validation and calibration of NAT-based assays.

The Committee endorsed the proposed project.

6.2.6 Babesia antibody

Babesiosis is a malaria-like illness that has been transmitted through blood transfusion. Identification of blood donors infected with the Babesia parasites may be possible by Babesia antibody tests; however, there are no antibody panels available to standardize and validate the tests. The Committee considered priorities to create a WHO Babesia antibody reference panel covering different major human and veterinary Babesia species worldwide (WHO/BS/10.2153). Currently, appropriate materials are not available in sufficient amounts and support from WHO would be required to access suitable materials. The initial focus would be B. microti. These materials may be very useful for assessment of antibody tests as they become available, e.g. for blood screening.

The Committee endorsed the project but noted that the workplan would be further clarified at the next meeting of the relevant WHO collaborating centres.

6.2.7 Chikungunya virus RNA for NAT-based assays

The Committee was informed that Chikungunya virus is transmitted between humans via mosquito bites and also via blood transfusion. Different episodes of outbreaks affecting different regions of the world had been reported in recent years. Currently there was no screening assay available to detect the viral RNA in blood products, for donor screening or for diagnosis of infection. The Committee considered a proposal to develop an International Chikungunya RNA Reference Reagent (WHO/BS/10.2153). In Europe, different laboratories
were currently developing Chikungunya NAT-based assays, to be prepared for a potential outbreak that might occur with the continuous presence of the insect vector in parts of Europe. Such a reagent may be used as a global standard for the development and validation of assays for the detection of viral RNA.

The Committee endorsed the project but noted that the workplan would be further clarified at the next meeting of the relevant WHO collaborating centres.

6.2.8  **Anti-HTLV I/II reference panel**

The Committee was provided with an update of an ongoing project. Efforts were in progress to acquire HTLV I- and HTLV II-positive plasma units for the design of a reference panel. Several plasma units had been obtained that may be fit for purpose. However, further consideration was being given to whether to include a broader diversity of well-characterized materials.

6.2.9  **Plasmodium falciparum antibody reference panel**

The Committee was reminded that the goal of this ongoing project was to create a reference panel that might be useful for evaluation and assessment of antibody tests. Information was provided on efforts to acquire antibody-positive plasma representing the four predominant human plasmodial infections. Suitable units with high-titre antibody to \textit{P. falciparum} had been obtained in Ghana. Efforts to obtain a sufficient amount of well-characterized material for other plasmodial species would continue.
7. International reference materials – biotherapeutics (other than blood products)

7.1 WHO International Standards and Reference Reagents – biotherapeutics (other than blood products)

7.1.1 Thyroid-stimulating antibody

Thyroid-stimulating hormone (TSH) receptor autoantibodies are important in the pathogenesis of autoimmune hyperthyroidism and comprise thyroid-stimulating antibodies (TSAbs) and thyroid-blocking antibodies. TSAbs can be detected in the vast majority of patients with hyperthyroidism caused by Graves disease and have also been associated with a small proportion of patients with toxic multinodular goitre. These autoantibodies interact with the TSH receptor on thyroid follicular cells and usually result in global stimulation of thyroid activity. Measurements of TSAbs are useful in the diagnosis and management of disease. For example, the detection of TSAbs has been used to predict relapse or remission in patients receiving treatment for Graves disease, and also to predict neonatal hyperthyroidism in children of mothers with Graves disease. The WHO First International Standard preparation, coded 90/672, consisted of freeze-dried plasma proteins from a single human patient with high TSAb levels, who was pregnant and whose plasma was regularly exchanged by plasmapheresis during pregnancy; 90/672 had been widely used for the calibration of assays to measure TSH receptor autoantibodies in human serum.

The Committee was informed that stocks of this preparation were now exhausted. In 2006, the Committee had recognized the need for a replacement International Standard for TSAbs for the calibration of these assays. The Committee considered a report (WHO/BS/10.2142) of the characterization of a candidate standard for TSAb in an international collaborative study carried out by 13 laboratories in 6 countries, and a comparison by receptor-binding assay and bioassay with the existing International Standard, coded 90/672. The mean estimate of the TSAb content of the candidate standard, coded 08/204, using receptor-binding assays was 0.113 IU per ampoule (95% confidence limits 0.106 to 0.120). The results of the study also indicated that the candidate standard was sufficiently stable to serve as an International Standard, with a predicted yearly loss of 0.018% when stored at –20 °C, on the basis of a thermally accelerated degradation study.

The Committee endorsed the proposal to establish the preparation 08/204 as the WHO Second International Standard for TSAb, with an assigned value of 113 mIU/ampoule. The Committee noted that the relationship between binding activity and stimulatory activity should be clarified in the “information for users”, and by consultation with users.
7.1.2 **Follicle-stimulating hormone, human, recombinant, for bioassay**

Follicle-stimulating hormone (FSH) is a glycoprotein hormone produced in the anterior pituitary gland, which plays a major role in the regulation of reproductive processes and pubertal maturation. Human FSH, initially of urinary origin, but now more commonly made by recombinant technology, has been widely used as a therapeutic product in the treatment of fertility disorders. The WHO First International Standard for recombinant human FSH (rhFSH), in ampoules coded 92/642, had been established in 1995 and had been widely used for the calibration of preparations of recombinant DNA-derived human FSH by bioassay. The global requirement for such a standard was evidenced by the increasing number of manufacturers involved in the production of therapeutic rhFSH. The Committee was informed that stocks of the WHO First International Standard were almost exhausted and there was an urgent requirement to replace the International Standard. A new preparation of rhFSH had been filled into ampoules (NIBSC Code 08/282), following procedures recommended by WHO, and an international collaborative study had been organized with expert laboratories to aid in the value assignment of the proposed WHO Second International Standard (WHO/BS/10.2140).

The Committee endorsed the proposal to establish the preparation 08/282 as the WHO Second International Standard for FSH, human, recombinant, for bioassay, 126 IU/ampoule. The Committee noted the need to extend stability studies to an assessment after 18 months’ storage, and to report back to the Committee when these data were available.

7.1.3 **Sex hormone-binding globulin**

Sex hormone-binding globulin (SHBG) is a 93.4 kDa homodimeric glycoprotein, synthesized by hepatocytes, which specifically binds sex steroid hormones such as testosterone and estradiol in the blood, with high affinity. The high-affinity binding of SHBG to sex steroid hormones limits the amount of free, biologically active hormone available to target cells, and thus SHBG plays an important role in regulating the actions of sex steroid hormones.

A number of pathological conditions can affect the circulating levels of SHBG, and serum measurements of SHBG are important in the diagnosis of conditions associated with abnormal sex steroid function, such as hyperandrogenism, infertility, polycystic ovary disease, impotence and abnormal liver and thyroid function. Methods for the measurement of SHBG are primarily based on immunoassay, although assays based on the specific binding of radiolabelled steroid hormones have been used historically.

The Committee was reminded that the WHO First International Standard for SHBG, coded 95/560, had been established in 1998 and had been widely used.
for the calibration of immunoassays and binding assays for the measurement of serum SHBG levels. Stocks of this WHO First International Standard were now almost exhausted and there was an urgent requirement to replace the standard.

An international collaborative study (WHO/BS/10.2141) was organized with expert laboratories to aid in the value assignment of the proposed WHO Second International Standard, and to assess the immunoreactivity and biological (androgen) binding activity of the candidate preparation by both SHBG immunoassay and SHBG-binding assay.

On the basis of the outcome of the study, the Committee endorsed a proposal to establish the preparation 08/266 as the WHO Second International Standard SHBG, and assigned a value of 180 IU/ampoule. The Committee further noted that, by convention, 1 IU SHBG was equivalent to 1 pmol.

7.1.4 **Granulocyte colony-stimulating factor**

The Committee was reminded that the current WHO First International Standard for granulocyte colony-stimulating factor (G-CSF) (88/502) had been established in 1992, with an assigned potency value of 10 000 IU. Stocks of this International Standard were now extremely low and a replacement was required. The WHO International Standard was primarily intended for the potency estimation of G-CSF products, which were used for several indications relating to neutropenia. Such uses included decreasing the incidence of infection, reducing the duration of neutropenia, e.g. febrile neutropenia in patients with nonmyeloid malignancies undergoing myeloablative chemotherapy followed by marrow transplantation, for reducing the time to neutrophil recovery and the duration of fever subsequent to chemotherapy in patients with acute myeloid leukaemia. In addition, G-CSF was used chronically in symptomatic patients with severe neutropenia (congenital, cyclic or idiopathic), for reducing the incidence and duration of sequelae of neutropenia, e.g. fever, infections, oropharyngeal ulcers. Increasingly, G-CSF was being used for mobilization of haematopoietic progenitor cells into the peripheral blood for collection by leukapheresis and haematopoietic stem-cell transplantation.

Five candidate preparations of human sequence recombinant G-CSF were formulated and lyophilized at NIBSC prior to evaluation in a collaborative study for their suitability to serve as a replacement WHO International Standard (WHO/BS/10.2133). The preparations were tested by 13 laboratories using in vitro bioassays. The candidate preparation, 09/136, was judged suitable to serve as a replacement WHO International Standard, based on the data obtained for biological activity and stability (predicted loss per year of less than 0.01% at the recommended storage temperature of –20°C).
The Committee endorsed the proposal to establish the preparation 09/136 as the WHO Second International Standard for G-CSF, with an assigned value of 95 000 IU/ampoule.

7.2 Proposed new projects – biotherapeutics (other than blood products)

7.2.1 WHO reference panel anti-erythropoietin antibodies testing

The Committee endorsed a proposed new project (WHO/BS/10.2153) on the development of a WHO reference panel for anti-erythropoietin antibodies, to be used in the diagnosis and study of iatrogenic immune responses to therapeutic erythropoietin.

7.2.2 Human proinsulin

The Committee endorsed a proposed new project (WHO/BS/10.2153) on the development of a replacement WHO International Standard for human proinsulin for immunoassay.

7.2.3 Human insulin C-peptide

The Committee endorsed a proposed new project (WHO/BS/10.2153) on the development of a replacement WHO International Standard for human insulin C-peptide for immunoassay.

7.2.4 Proposed WHO First Reference Reagent for human interleukin-29

The Committee endorsed a proposed new project (WHO/BS/10.2153) on the development of a WHO Reference Reagent for human interleukin (IL)-29.

7.2.5 CD4+ cell counting standard

The Committee endorsed a proposed new project (WHO/BS/10.2153) on the development of a WHO Reference Reagent to serve as a standard for CD4+ cell counting or sorting methods. The Committee also noted a need to evaluate the likely public health benefit from extensions of this new area of activity to cover other widely measured cell phenotypes.

7.3 Ongoing stability monitoring – biotherapeutics (other than blood products)

7.3.1 Somatropin (recombinant human growth hormone)

The Committee noted a report (WHO/BS/10.2135) of physicochemical stability data on the WHO Second International Standard for somatropin (preparation code 98/574). Eleven years after being filled, ampouled materials that had been
stored at −20 °C were compared with frozen baseline samples stored continuously at −150 °C. No differences were found in either monomer content (which predicted biological activity) or HPLC purity profile.

The Committee noted that real-time stability studies had confirmed predictions at the time of establishment, and endorsed a proposal that the material could be considered stable until replacement, or 20 years after filling, whichever was the sooner.
8. International reference materials – antibiotics

8.1 WHO International Standards and Reference Reagents – antibiotics

8.1.1 Vancomycin

Vancomycin is a mixture of tricyclic glycosylated peptides and is produced by fermentation from *Amycolatopsis orientalis*. The antibiotic inhibits cell wall synthesis in Gram-positive bacteria and may be used as a drug of “last resort”. The WHO First International Standard for vancomycin was established in 1964, with an assigned activity of 1007 IU/mg (approximately 100 mg/vial). The Committee was informed that a replacement batch was needed, owing to low stocks.

A candidate replacement material was obtained, evaluated in preliminary studies, and then studied in an international collaborative study (WHO/BS/10.2151). A total of 12 laboratories participated, from Algeria, Argentina, Canada (2), China, the Czech Republic, France, Malaysia, Portugal, the Russian Federation, Thailand and the Council of Europe (EDQM).

On the basis of the results of the study, the Committee endorsed the proposal to establish the preparation ISA_39036 as the WHO Second International Standard for vancomycin, with an assigned antimicrobiological activity of 109 700 IU per vial. The Committee also recommended that real-time stability data be submitted for its review after a suitable interval in the future.

8.2 Proposed new projects – antibiotics

8.2.1 Dihydrostreptomycin

The Committee endorsed the proposed new project (WHO/BS/10.2153) on the development of a replacement WHO International Standard for dihydrostreptomycin.
References


Annex 1

WHO Recommendations, Guidelines and other documents related to the manufacture and control of biological substances used in medicine

The Recommendations (previously called Requirements) and Guidelines published by the World Health Organization are scientific and advisory in nature but may be adopted by a national regulatory authority as national requirements or used as the basis of such requirements.

These international Recommendations are intended to provide guidance to those responsible for the production of biologicals, as well as to others who may have to decide upon appropriate methods of assay and control to ensure that these products are safe, reliable and potent.

Recommendations concerned with biological substances used in medicine are formulated by international groups of experts and are published in the Technical Report Series of the World Health Organization as listed here. A historical list of Requirements and other sets of Recommendations is available on request from the World Health Organization, 20 avenue Appia, 1211 Geneva 27, Switzerland.

Reports of the Expert Committee on Biological Standardization published in the WHO Technical Report Series can be purchased from:

WHO Press
World Health Organization
20 avenue Appia
1211 Geneva 27
Switzerland
Telephone: +41 22 791 3264
Fax: +41 22 791 4857
E-mail: bookorders@who.int
Web site: http://www.who.int/bookorders

Individual Recommendations and Guidelines may be obtained free of charge as offprints by writing to:

Department of Essential Medicines and Health Products
World Health Organization
20 avenue Appia
1211 Geneva 27
Switzerland

\[1\] Abbreviated in the following pages as TRS.
<table>
<thead>
<tr>
<th>Recommendations, Guidelines and other documents</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acellular pertussis component of monovalent or combined vaccines</td>
<td>Adopted 1996, TRS 878 (1998)</td>
</tr>
<tr>
<td>Animal cells, use of, as in vitro substrates for the production of biologicals</td>
<td>Revised 2010, TRS 978 (2013)</td>
</tr>
<tr>
<td>Biological products prepared by recombinant DNA technology</td>
<td>Adopted 1990, TRS 814 (1991)</td>
</tr>
<tr>
<td>Biological standardization and control: a scientific review commissioned by the UK National Biological Standards Board (1997)</td>
<td>Unpublished document WHO/BLG/97.1</td>
</tr>
<tr>
<td>Biotherapeutic products, similar</td>
<td>Adopted 2009, TRS 977 (2013)</td>
</tr>
<tr>
<td>Diphtheria, tetanus, pertussis and combined vaccines</td>
<td>Revised 1989, TRS 800 (1990); Addendum 2003, TRS 927 (2005); Addendum 2005, TRS 941 (2007)</td>
</tr>
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<td>Recommendations, Guidelines and other documents</td>
<td>Reference</td>
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<td>------------------------------------------------</td>
<td>-----------</td>
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<tr>
<td>Haemorrhagic fever with renal syndrome (HFRS) vaccine (inactivated)</td>
<td>Adopted 1993, TRS 848 (1994)</td>
</tr>
<tr>
<td>Hepatitis B vaccine prepared from plasma</td>
<td>Revised 1987, TRS 771 (1988)</td>
</tr>
<tr>
<td>Hepatitis B vaccines made by recombinant DNA techniques</td>
<td>Revised 2010, TRS 978 (2013)</td>
</tr>
<tr>
<td>Human interferons prepared from lymphoblastoid cells</td>
<td>Adopted 1988, TRS 786 (1989)</td>
</tr>
<tr>
<td>Influenza vaccine (inactivated)</td>
<td>Revised 2003, TRS 927 (2005)</td>
</tr>
<tr>
<td>Influenza vaccine (live)</td>
<td>Revised 2009, TRS 977 (2013)</td>
</tr>
<tr>
<td>Influenza vaccines, human, pandemic, regulatory preparedness</td>
<td>Adopted 2007, TRS 963 (2011)</td>
</tr>
<tr>
<td>Japanese encephalitis vaccine (inactivated) for human use</td>
<td>Revised 2007, TRS 963 (2011)</td>
</tr>
<tr>
<td>Measles, mumps and rubella vaccines and combined vaccine (live)</td>
<td>Adopted 1992, TRS 848 (1994); Note TRS 848 (1994)</td>
</tr>
<tr>
<td>Meningococcal A conjugate vaccines</td>
<td>Adopted 2006, TRS 962 (2011)</td>
</tr>
<tr>
<td>Meningococcal C conjugate vaccines</td>
<td>Adopted 2001, TRS 924 (2004); Addendum (revised) 2007, TRS 963 (2011)</td>
</tr>
<tr>
<td>Papillomavirus vaccine, human</td>
<td>Adopted 2006, TRS 962 (2011)</td>
</tr>
<tr>
<td>Recommendations, Guidelines and other documents</td>
<td>Reference</td>
</tr>
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<td>-----------</td>
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<tr>
<td>Pneumococcal conjugate vaccines</td>
<td>Revised 2009, TRS 977 (2013)</td>
</tr>
<tr>
<td>Regulation and licensing of biological products in countries with newly developing regulatory authorities</td>
<td>Adopted 1994, TRS 858 (1995)</td>
</tr>
<tr>
<td>Synthetic peptide vaccines</td>
<td>Adopted 1997, TRS 889 (1999)</td>
</tr>
<tr>
<td>Thromboplastins and plasma used to control oral anticoagulant therapy</td>
<td>Revised 1997, TRS 889 (1999)</td>
</tr>
<tr>
<td>Recommendations, Guidelines and other documents</td>
<td>Reference</td>
</tr>
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<td>-----------------------------------------------------------------------------------------------------------------</td>
<td>-----------------------------------------------</td>
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<td>Transmissible spongiform encephalopathies, WHO tables on tissue infectivity distribution</td>
<td>Revised 2010, WHO (2010)</td>
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<td><a href="http://www.who.int/bloodproducts/">http://www.who.int/bloodproducts/</a></td>
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<td>tablestissueinfectivity.pdf</td>
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<tr>
<td>Tuberculins</td>
<td>Revised 1985, TRS 745 (1987)</td>
</tr>
<tr>
<td>Typhoid vaccine</td>
<td>Adopted 1966, TRS 361 (1967)</td>
</tr>
<tr>
<td>Vaccines, lot release</td>
<td>Adopted 2010, TRS 978 (2013)</td>
</tr>
<tr>
<td>Vaccines, preclinical evaluation</td>
<td>Adopted 2010, TRS 978 (2013)</td>
</tr>
<tr>
<td>Vaccines, stability evaluation</td>
<td>Adopted 2006, TRS 962 (2011)</td>
</tr>
<tr>
<td>Yellow fever vaccine</td>
<td>Revised 2010, TRS 978 (2013)</td>
</tr>
<tr>
<td>Yellow fever vaccine, laboratories approved by WHO for the production of</td>
<td>Revised 1995, TRS 872 (1998)</td>
</tr>
<tr>
<td>Yellow fever virus, production and testing of WHO primary seed lot 213-77 and reference batch 168-73</td>
<td>Adopted 1985, TRS 745 (1987)</td>
</tr>
</tbody>
</table>
Annex 2

Guidelines for independent lot release of vaccines by regulatory authorities

Abbreviations

1. Introduction
   1.1 Scope

2. Glossary

3. General considerations
   3.1 Considerations for establishing lot release procedures by the NRA/NCL
   3.2 Encouragement of networking and work-sharing

4. Responsibilities of the NRA/NCL and manufacturer in lot release
   4.1 Responsibility of the NRA/NCL in lot release
   4.2 Responsibility of the manufacturer in NRA/NCL lot release
   4.3 Establishment of quality management systems for the NRA/NCL

5. Conducting lot release
   5.1 Protocol review
      5.1.1 Principles
      5.1.2 Summary protocol template
      5.1.3 Checklist for protocol review
      5.1.4 Protocol review process
      5.1.5 Handling discrepancies and OOS results in summary protocols
   5.2 Independent testing
      5.2.1 Purpose of independent testing
      5.2.2 Prerequisites for setting up independent testing for lot release
      5.2.3 Establishment of a testing policy
      5.2.4 Criteria for selection of tests for lot release and percentage of lots to be tested
      5.2.5 Importance of reference preparations for lot release
      5.2.6 Standards
      5.2.7 Practical considerations
      5.2.8 Release specifications
      5.2.9 Evaluation of NCL results

6. Data monitoring
   6.1 Trend analysis including data from the NCL
   6.2 Comparison of results of the manufacturer with those of the NCL

7. Evaluation of the lot and the decision-making process
   7.1 Establishment of decision-making procedures
Recommendations published by WHO are intended to be scientific and advisory. Each of the following sections constitutes guidance for national regulatory authorities (NRAs) and for manufacturers of biological products. If an NRA so desires, these recommendations may be adopted as definitive national requirements, or modifications may be justified and made by the NRA. It is recommended that modifications to these recommendations be made only on condition that the modifications ensure that the vaccine is at least as safe and efficacious as that prepared in accordance with the recommendations set out below. The parts of each section printed in small type are comments for additional guidance intended for manufacturers and NRAs, which may benefit from these details.
**Abbreviations**

AEFI  
adverse events following immunization

BCG  
bacille Calmette–Guérin

DTP  
diphtheria–tetanus–pertussis vaccine

GMP  
good manufacturing practices

HPV  
human papilloma virus

MMR  
measles, mumps and rubella vaccine

NCL  
national control laboratory

NRA  
national regulatory authority

OOS  
out of specification

OPV  
oral poliomyelitis vaccine

PMS  
post-marketing surveillance

QMS  
quality management system

SOP  
standard operating procedure

USA  
United States of America

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**1. Introduction**

The lot release of vaccines by regulatory authorities is part of the regulation of vaccines and involves the independent assessment of each lot of a licensed vaccine before it is released on to the market. This assessment is based, as a minimum, on the review of manufacturers’ summary protocols. It may be supplemented by other documents such as the release certificate from the responsible national regulatory authority (NRA) or national control laboratory (NCL) and, in some circumstances, by testing that is independent of the manufacturers’ quality-control testing.

WHO provides support for lot release programmes through the provision of written standards and measurement standards, strengthening the lot release function of NRAs and providing training (1–4). However, a need for further guidance was identified at a WHO consultation held in Ottawa in 2007.

This document provides recommendations and strategies for the lot release of vaccines by the NRAs/NCLs of producing and procuring countries. It should be read in conjunction with the recommendations/guidelines for specific products (e.g. recommendations for bacille Calmette–Guérin (BCG), oral...
poliomyelitis (OPV), measles, mumps and rubella (MMR), diphtheria–tetanus–pertussis (DTP), human papilloma virus (HPV) and rotavirus vaccines) (5–10).

Although it is difficult to provide a set of guidelines that apply to all national situations, an attempt has been made to cover a range of acceptable possibilities. Independent lot release involves the confirmation that each lot meets the specifications in the approved marketing authorization for the product. Under defined circumstances, laboratory testing by an NCL can provide added value to this confirmation. The need for testing should, however, be justified according to the criteria specified in this document and the laboratory should operate under an appropriate quality assurance system. When independent laboratory testing is undertaken, NCLs should ensure that it is conducted according to the principles defined in this document. Testing under inappropriate conditions may generate inaccurate data and lead to incorrect decisions. These Guidelines also highlight the importance of networking and work sharing among NRAs/NCLs.

The Guidelines are intended to serve as a guide for national requirements for lot release. If an NRA wishes, the Guidelines may be adopted as definitive national requirements, or modifications may be justified and made by the NRA. It is recommended that modifications to the principles and technical specifications of the Guidelines should be made only if the modifications ensure that the risks of introducing vaccines for use in public health programmes are no greater than as outlined in the Guidelines.

1.1 Scope
This document focuses on vaccines for human use. However, the main principles can also be applied to other biologicals.

The document is intended to provide guidance to the NRAs/NCLs and to vaccine manufacturers. It may also be relevant to public health authorities such as a national immunization programme.

2. Glossary
The definitions given below apply to the terms as used in these guidelines. The terms may have different meanings in other contexts.

Deviation: departure from a standard, norm or set of limits.

Lot/sub-lot: a defined quantity of starting material, packaging material or product, processed in a single process or series of processes so that the quantity is expected to be homogeneous. It may sometimes be necessary to divide a lot into a number of sub-lots, which are later brought together to form a final homogeneous lot. In continuous manufacture, the lot must correspond to a defined fraction of the production, characterized by its intended homogeneity. The lot size can be defined either as a fixed quantity or as the amount produced in a fixed time period.
Lot release: the process of NRA/NCL evaluation of an individual lot of a licensed vaccine before giving approval for its release on to the market.

Marketing authorization: an official document issued by the competent NRA for the purpose of marketing or free distribution of a product after evaluation for safety, efficacy and quality.

Non-compliance: failure or refusal to comply with a standard or a set of limits.

Out of specification (OOS): an OOS result is generated when a vaccine is tested and fails to meet a predefined specification.

Responsible NRA/NCL: the NRA/NCL taking responsibility for regulatory oversight of a product with regard to the critical regulatory functions defined by WHO, including independent lot release. The responsible NRA/NCL is usually that of the country of manufacture, unless specific agreements exist within defined territories, such as in the European Union, where the “country” of manufacture is the European Union and the activity of the responsible NRA/NCL is designated from among the Member States.

Self-procured vaccine: a vaccine that is procured directly from a source outside the country without the intervention of WHO/United Nations procurement programmes.

Source material/starting material: any substance of a defined quality used in the production of a vaccine product, but excluding packaging materials.

Summary protocol: (also called “lot summary protocol”) a document summarizing all manufacturing steps and test results for a lot of vaccine, which is certified and signed by the responsible person of the manufacturing company.

Yearly biological product report: a report submitted annually by manufacturers to the NRA/NCL, containing production information on both bulk and final lots, including test methods and results and reasons for any recalls and corrective action taken, as well as other pertinent post-marketing information.

3. General considerations

Vaccines are biological products used in healthy populations. The impact of using substandard lots may not be known for a very long time (years). Similarly, safety issues with a particular lot may not be known immediately (within a few hours) after administration, and there could be a drastic impact if a large number of healthy persons receive a vaccine before a problem is recognized. For these reasons, a careful independent review of manufacturing and quality-control data on every lot is necessary before it is marketed. Problems regarding vaccine quality have a direct impact on the public acceptance of immunization programmes, thus potentially compromising public health strategies. Consequently, it is essential to assure the consistent quality of each lot before it is released onto the market.
Furthermore, vaccines and many of the tests applied to them are of a biological and complex nature, and have an inherent potential for variability. Therefore, an independent review of critical data from each lot of vaccines is essential to assure the consistent quality of each manufactured lot.

Reference standards used in the testing of vaccines are also biological in nature and prone to the same issues of complexity and stability as the vaccines themselves. For new products, national or international standards or reference preparations are not always available and there may be limited data on the stability of in-house or working standards used. Independent review of data is necessary in order to gain confidence in the results of tests using these preparations.

It is strongly recommended that NRAs/NCLs ensure that there is independent testing and lot release for vaccines used in their country, either based on their own evaluation, using as a minimum a thorough review and approval of the manufacturers’ summary protocol (for details see section 5.1), or through recognition of the decision of another regulatory authority.

All vaccine lots should be released by an NRA/NCL; however, in defined exceptional circumstances such as a public health emergency, exemption could be allowed. The permitted circumstances and the procedures to be followed to ensure quality in the absence of lot release should be covered by legal provisions.

Lot release is part of the whole regulatory framework, which includes marketing authorization, good manufacturing practices (GMP) inspection and post-marketing surveillance (PMS). The relationship between the NRA and the NCL varies from country to country, but in all cases it is essential that the different branches of the regulatory structure interact and exchange information effectively.

Each country should establish the national guidelines for lot release that define all procedures, from the submission of the lot for release to the issue of lot release certificates. The principles found in this document may assist in the development of these national guidelines.

3.1 Considerations for establishing lot release procedures by the NRA/NCL

Current approaches to conducting lot release of vaccines include review of the summary protocol only, review of the summary protocol with independent testing (full or selected testing), and recognition/acceptance of lot release certificates from the responsible NRA/NCL. These approaches are not mutually exclusive and different approaches may be used for different products in the same country.

It is the responsibility of the NRA/NCL to decide on an appropriate strategy for each vaccine, taking into consideration the nature of the vaccine, the post-marketing experience (including production history and safety profile), and the availability of other independent evidence of product quality (see section 5.2). In some cases, the same lot may be used to supply multiple countries. Multiple
testing can be costly and time consuming. In addition, many biological assays are highly variable, and repetitive testing can result in “false” OOS results, which then require extensive investigation and delay vaccine supply. The decision to repeat tests on a lot that has already been tested by another competent authority should be carefully considered in light of all available information.

For vaccines produced and authorized in a country, either for domestic use or for export, the NRA of the country should take the responsibility for regulatory oversight of vaccine quality. The NRA/NCL should initially test the vaccine, in addition to carrying out a critical review of the summary protocols. After confirmation of the consistency of the quality through testing the chosen parameters, release of further lots should include full or selected testing or no testing, depending on the nature of the product and established experience. In the case of a vaccine not licensed in the country of manufacture, the NRA that granted the marketing authorization should take full responsibility for regulatory oversight. However, cooperation with the NRA of the producing country is recommended.

For self-procured vaccines, the procuring NRA/NCL may consider alternative approaches to be acceptable for assuring the safety and quality of these products. As a minimum, review of the summary protocol is essential. Independent tests may be useful, depending on the history of production, the nature of the product (see section 5.2.3) and the capacity of the NCL. Recognition/acceptance of lot release certificates from the NRA/NCL of the country where the vaccine is manufactured, or from another competent NRA/NCL, should also be considered as an alternative (see section 7.1).

For vaccines supplied through United Nations agencies, further release by the NRA/NCL of receiving countries is not recommended (see section 7.2) because such products are prequalified by WHO and released by the responsible NRA/NCL.

3.2 Encouragement of networking and work-sharing

Regional laboratory networks can serve as a forum for sharing information, exchanging experience on technical issues and facilitating assistance between NRAs/NCLs. It is recommended that WHO regional offices take the lead in establishing regional laboratory networks in areas where these have not yet been developed. It would be useful to have a forum in the regional network for sharing information on lots that were found to be OOS, and this would also be beneficial on a global level.

Development of a network expands the capacity of individual NRAs/NCLs beyond their own limits, through work-sharing, and ideally, by building confidence in the evaluation performed by other network members, avoids the same lot being tested unnecessarily and repeatedly by different NCLs. The
sharing of test results can contribute to reducing the number of animals used for testing and can prevent samples being tested in laboratories that perform certain assays only infrequently, and so may have problems in maintaining technical competence. Work-sharing also enables the development of more complex and specialized methods through repetition of tasks and it provides a support network for problem solving.

Establishing networks would be part of the capacity-building activities for countries in a region. A fully functional regional laboratory network is a long-term goal, but cooperation can begin in the short term, with sharing of scientific information and experiences with methodologies regarding the evaluation and release of different products. Meetings should be organized periodically to promote transparency and mutual confidence between the NRAs/NCLs.

Although full mutual recognition of lot release certificates among NRAs/NCLs would be ideal, this is a complex issue, with a number of difficulties in practice. Nevertheless, an effective regional network can help build the foundations for achieving such a goal.

4. Responsibilities of the NRA/NCL and manufacturer in lot release

The quality, safety and efficacy of a medicinal product such as a vaccine are the responsibility of the manufacturer. The regulatory authority of the country is responsible for establishing procedures to ensure that this responsibility is met.

The same requirements of regulatory oversight should apply to the production of vaccines, whether they are intended for domestic use or for export.

4.1 Responsibility of the NRA/NCL in lot release

Marketing authorization for a vaccine should be granted by an NRA, which should also be responsible for continued post-authorization monitoring. In carrying out these activities, the NRA should have access to expert advice and laboratory facilities. The activities of the NRA should be backed by legislation, which should include provisions for lot release.

An NRA/NCL that undertakes a lot release programme should have sufficient capacity and expertise to evaluate lot release protocols effectively. Timelines and responsibilities of the NRA/NCL for issuing the lot release certificate should be defined as part of the legal provisions. The manufacturer and relevant health authorities should be informed in the event of a delay.

The NRA/NCL should have the authority to request appropriate samples from manufacturers when required. The samples should be properly identified and portions may be kept for future reference.
Where independent testing is required, the NRA/NCL should have the capacity to perform the appropriate tests on all relevant samples (which may include critical upstream components, bulk and finished products) or have access to a laboratory that is competent in the tests. This would require that the NRA/NCL has access to specialized facilities, equipment and expertise. The NCL should be independent of the manufacturer, and staff should not be shared. In particular, there should be a clear separation of lot release activities in cases where the NCL and manufacturer share a site.

The NRA/NCL should ensure that the mechanism for the independent lot release procedure is made public in a clear and transparent way regarding requirements and timelines, so that the process is completed smoothly and in a timely manner.

NRAs/NCLs of producing/releasing countries have the responsibility to provide information concerning the quality of the lot of a product to the NRA/NCL of an importing country, upon request. Rules and procedures regarding confidentiality of information should be established and the data submitted by manufacturers and other NCLs/NRAs should be kept confidential unless agreed otherwise.

The NRA/NCL of a producing/releasing country has the responsibility to ensure the production and release of vaccines of assured quality whether they are used within the country or exported. Vaccines for local use and those for export should have the same level of quality.

4.2 **Responsibility of the manufacturer in NRA/NCL lot release**

The manufacturer has a number of responsibilities in terms of NRA/NCL lot release. In this regard, the manufacturer should:

- collaborate with the responsible NRA/NCL to develop the product summary protocol template when requested (the WHO summary protocol of each product could be used as the template);
- submit each manufacturing and control summary protocol;
- if requested, submit samples in an appropriate condition, including packaging, leaflet and label;
- assist the responsible NRA/NCL in technical transfer of testing methods;
- submit the lot release certificate of the responsible NRA in the case of export products;
- provide product-specific reagents and working reference materials, as needed;
- participate in collaborative studies in establishment of a national standard;
- work with NRA/NCL to resolve any discrepancy in test results;
- take appropriate action on any issues related to error or non-compliance;
- take appropriate action on any rejected lots according to GMP requirements (11);
- provide any documents or other information regarding the quality of the vaccine, as required by the NRA/NCL.

4.3 Establishment of quality management systems for the NRA/NCL

A quality management system (QMS) should be in place to support lot release activities. The QMS system should include the following key elements: trained and qualified personnel, management of records and documentation, identification and retention of samples (when applicable), use of validated test procedures, written procedures, internal and external audit systems, and oversight procedures. The recommendations in the WHO Guidelines for national authorities on quality assurance for biological products should be applied (1).

5. Conducting lot release

The manufacturer’s summary protocol should be reviewed by an NRA/NCL, to ensure that specifications defined in the marketing authorization dossier are met before release of a lot on to the market. Product consistency should be assessed through trend analysis on successive lots (see section 6). Where NCLs do not receive consecutive lots, or receive only a small number of the production lots, interpretation of trend may require additional information (e.g. yearly biological product report). Where appropriate, review of the summary protocol can be complemented by independent testing. In the case of imported vaccines, any available lot release certificate issued by the responsible NRA/NCL, particularly the one from the producing country, should be considered in the overall assessment of a vaccine lot. If the lot release certificate is not provided together with the summary protocol, the NRA/NCL should have the authority to request it.

A need for independent testing should be carefully considered in the establishment of the lot release procedures. Assessment of vaccine lots by an NCL can add value to the information provided in the summary protocol, if the testing is performed by experienced, competent and skilled laboratory staff supported by a QMS and appropriate laboratory facilities.

5.1 Protocol review

Manufacturers’ summary protocols summarize information taken from the production and test records, according to GMP requirements, to ensure that the
lot meets the specifications in the market authorization. In addition, summary protocols submitted to the NRA/NCL should be approved by the person designated as responsible for quality assurance or quality control of the manufacturer. In general, the format and content of the protocol is finalized and approved by the NRA/NCL during the review of the licence application. The format of the protocol should be amended in response to changes in the approved production process and should be approved by the NRA/NCL.

5.1.1 **Principles**

Protocol review is conducted by qualified NRA/NCL staff. As far as possible, the format of the summary protocol of a specific product should be the same in different markets. However, the format of a summary protocol can vary with respect to additional information required by the NRA of an importing country.

An independent review of critical data from each lot of vaccines is essential, in order to:

- assure the consistency of quality of each manufactured lot;
- obtain confidence in the claimed strength of active components;
- assess the validity and accuracy of the tests performed.

This review encompasses the traceability of critical source materials, active and critical components used in the manufacture of the product, and the results from tests performed by the manufacturer at various stages of production, including tests performed on critical components, intermediates, final bulk and final product.

5.1.2 **Summary protocol template**

Since protocol review is an essential component of the lot release process, it is crucial that the template of the summary protocol is developed carefully on the basis of the approved marketing authorization dossier, and approved by the NRA/NCL. WHO templates are available for some vaccines, but the agreed protocol should also take into account specific requirements in the marketing authorization approved for the product. Any changes to the template due to changes in the manufacturing process or testing should be traceable. The template should be a controlled document and the manufacturer should not change it without the approval of the regulatory authority. It is important that NRA/NCL staff responsible for reviewing these documents ensure that the latest version of the licence is reflected in the summary protocol submitted by the manufacturer.

Each summary protocol is product specific, but there are a number of general items (see Table A2.1) that a summary protocol should cover.
### Table A2.1
Information to be included in the summary protocol for review

<table>
<thead>
<tr>
<th>Items</th>
<th>Essential information to cover</th>
<th>Critical parameters to review</th>
</tr>
</thead>
<tbody>
<tr>
<td>Identity of manufacturer</td>
<td>Name of manufacturer</td>
<td>Traceability and identity</td>
</tr>
<tr>
<td>Licence number</td>
<td>Unique licence number</td>
<td>Traceability and identity</td>
</tr>
<tr>
<td>Site(s) of manufacturing</td>
<td>Site of manufacturing for each bulk, final bulk and final product</td>
<td>Traceability and identity</td>
</tr>
<tr>
<td>Name and lot number</td>
<td>Name and lot numbers of the final products, bulk, final bulk and the diluent if applicable</td>
<td>Unique, systematic, traceability and identity</td>
</tr>
<tr>
<td>Lot size</td>
<td>Volume, number of doses and type of container</td>
<td>Listed information should fit within allowed parameters</td>
</tr>
<tr>
<td>Expiry dates</td>
<td>For each starting material (if applicable), intermediates, final bulk and final product</td>
<td>Expiry date of each component fits the shelf-life of the final product</td>
</tr>
<tr>
<td>Dates of manufacturing</td>
<td>For each critical starting material (e.g. seed lots, cell banks, starting materials of animal origin etc.), intermediate, final bulk and final product</td>
<td>Compared against noted expiry dates etc., to calculate and confirm values</td>
</tr>
<tr>
<td>Flowchart</td>
<td>Flowchart for traceability of the manufacturing process for major components, including lot numbers</td>
<td>Identity and logic flow for starting materials, intermediates, final bulk and final product confirmed</td>
</tr>
<tr>
<td>Strains and cell substrates</td>
<td>Name, seed lot number, passage number</td>
<td>Strain of production seed and type of cell substrate, lot/bank number, passage number of master and/or working lot/bank are the same as the one approved by the NRA on the marketing authorization and/or recommended by WHO (e.g. OPV) (6)</td>
</tr>
</tbody>
</table>
Table A2.1 continued

<table>
<thead>
<tr>
<th>Items</th>
<th>Essential information to cover</th>
<th>Critical parameters to review</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manufacturing process</td>
<td>Each production process (such as cultivation, purification, inactivation), the methods of quality-control tests as well as their release specifications and the results obtained; the lot number of intermediates and their size/volume, storage conditions</td>
<td>Confirm they are the same as the approved ones; yields of critical production processes are within the acceptable range</td>
</tr>
<tr>
<td>Formulation</td>
<td>Amount of active components in the final formulations, with the lot numbers and volumes of bulk concentrates; storage conditions</td>
<td>Verify calculated and actual values based on information provided</td>
</tr>
<tr>
<td>Quality-control tests</td>
<td>Actual results of tests on critical starting materials, intermediates, final bulk and final product and the specification; include the individual tests and the mean value; provide the starting date of the test, method, and a list of reference preparations, standards, critical reagents and their qualification status, plus the performance of relevant reference preparations, standards and internal controls, such as results of assay validity criteria (e.g. slope, intercept, linearity, 50% end-points, results of internal controls, challenge doses); provide statistical results, such as mean, geometric mean, standard deviation, 95% confidence intervals, etc., if applicable; include results of failed tests or note invalid tests if a test has been repeated</td>
<td>Demonstrate that the identity, purity, safety, potency (strength) and thermostability of the product are in compliance with the approved specifications; monitor the performance of reference material/test</td>
</tr>
</tbody>
</table>
5.1.3 **Checklist for protocol review**

The use of checklists in the review of protocols is highly recommended to ensure a complete and thorough review. A checklist should be developed for each section of the protocol, to ensure a complete review of the information. Checklists are usually developed according to the critical parameters in the production and control processes – such as the strain and acceptable passage level of seed, acceptable passage level of cell substrate, purification method, methods and release specifications of quality-control tests, and shelf-life of intermediates. Checklists are specific to a registered product and/or a test, in accordance with both the marketing authorization dossier and WHO recommendations, and may be a copy of the protocol template with the specific required manufacturing information included for reference (e.g. name of the cell line, origin, testing methods and specifications for starting materials, intermediates, final bulk and final product).

5.1.4 **Protocol review process**

The value of the protocol review process depends on the quality of the information provided by the manufacturer in the summary protocols. Reviewing summary protocols requires a good understanding of the product and of laboratory control methods. A summary protocol for a product can be reviewed by one person, or by a team of experts, depending on the complexity of the product and the structure of the NRA/NCL. Validated software, with adequate access controls and traceability for tracking and trending of the data submitted, may be useful for performing a meaningful review of protocols.

The lot release process starts with receipt of the manufacturer’s protocol and test samples, if required, and/or examples of the final label. After initial verification of the label information for the test sample and on the protocol, the protocols are logged into a database or otherwise recorded. At receipt, the first step in protocol review should be to confirm that the manufacturer has used the approved template for the given vaccine. Then the protocols are routed to individuals within the NRA/NCL who have already been identified on the basis of their expertise. This should be traceable according to QA management procedures.

If databases are used to capture information for a particular test or section of the protocol, these should already be in place before starting the review process. Databases on lot size, results of tests, performance of reference standards and controls, and so on are useful for tracking and trending of information. The results of tests and performance characteristics of reference standards and controls and specification limits, including appropriate confidence intervals of typical results for a period of time, should be shown. In all cases, databases should be secured to avoid unauthorized addition, revision or deletion of information, and a back-up system should be provided. A separate procedure should be developed for tracking
and trending of manufacturers’ results and the parameters to be tracked and trended, frequency of periodic reviews, actions to be taken in case of out-of-normal trends, etc.

In general, a particular lot of the product is satisfactory if the protocol review shows that all of the elements described in Table A2.1 have been compared against the characteristics approved in the marketing authorization and have been found to be compliant.

In some countries, for freeze-dried vaccines, the protocol or certificate of analysis of the particular lot of diluent is reviewed. However, this is not done in other countries, since diluents are not considered on their own to be biologicals.

5.1.5 **Handling discrepancies and OOS results in summary protocols**

Any discrepancies, errors or OOS results found in the summary protocol submitted should be documented and verified before they are communicated to the manufacturer. A procedure to communicate these issues should be developed by the NRA/NCL. This may include formal notification by memo or letter, an e-mail or minutes of telephone discussions. Manufacturers’ responses should be reviewed and documented in making the decision on the lot. This can include submission by the manufacturer of the corrected page/version of the summary protocol, which then should be traced by the NRA/NCL. Depending upon the nature and severity of the discrepancies or errors, the manufacturer may be asked to perform an investigation to determine the root cause of the issues, including steps for corrective and preventive actions to avoid similar problems in the future. For imported lots, communication with the NRA of the producing/releasing country may be required. For producing/releasing countries, communication with the country inspectorate may be required. Such information exchange can help to judge the corrective and preventive actions introduced by the manufacturer.

5.2 **Independent testing**

Independent testing enables the NCL to monitor key product parameters and the consistency of production on the basis of its own data. The development of NCL technical expertise also enables other issues regarding quality control of products to be independently assessed when they arise.

If quality testing is performed by a laboratory other than the NCL, the laboratory should be contracted, information exchange should be handled in a confidential manner, and there should be a system to ensure that there is no conflict of interest. The qualification of the laboratory should be assessed, and the performance of the laboratory testing should be evaluated by the NRA/NCL.
according to WHO recommendations (2). The final decision on the test results lies with the responsible NRA/NCL.

5.2.1 Purpose of independent testing

A lot release testing programme allows NCLs to verify the test results of manufacturers. When testing is performed in a systematic way by a qualified NCL, it can help to monitor the continuing suitability of the methods and reference materials and allow detection of possible drifts in these parameters that are unaccounted for. This can serve as feedback to the marketing authorization, in case a need is identified to revise the specification in the marketing authorization dossier, and the expertise can be used to aid GMP inspectors in a coordinated approach. Testing by NCLs also maintains independent expertise in the test methods. This is important for the overall competence of an NCL in effectively monitoring the product.

5.2.2 Prerequisites for setting up independent testing for lot release

A defined strategy for testing needs to be established as part of the overall policy on lot release. Knowledge of the marketing authorization dossier is essential for identifying and assessing the critical parameters for testing. Ideally, the NCL staff should be involved in the marketing authorization evaluation process (at least so far as concerns information on pharmaceutical quality).

A good QMS is essential when setting up a testing policy. The QMS should include a quality assurance system that is appropriate for testing laboratories, that is based on internationally recognized quality standards, and that undergoes regular internal and external review (see WHO guidelines (1)).

This would include aspects of technical staff training, maintenance of equipment, standard operating procedures (SOPs) for techniques, daily running of the system, and dealing with OOS results. The NCL should have sufficient skilled, trained and qualified personnel with the appropriate technical and scientific expertise, and appropriate equipment and infrastructure should be available.

Relevant test methods should be validated following quality assurance standards (including equipment qualification) if independent testing has to be performed. It is also necessary to establish documented and approved procedures and guidelines, both for internal use and for transparency with regard to partners, including other NCLs and the manufacturer of the product.

While not necessarily a prerequisite, good communication with the manufacturer of the product is an important element in developing an effective system. NCLs should discuss with the manufacturer the transfer of assays, if required. This should begin as early as possible in the marketing authorization process, to allow for transfer and qualification/validation of the methodology prior to application to the first lot for lot release testing. Since specifications
for some biological assays (e.g. potency, purity) are dependent on the analytical technique used, comparison of testing results between the NCL and the manufacturer is important to avoid potential discrepancies that may be related to the methodology used and not to the quality of the product.

### 5.2.3 Establishment of a testing policy

Implementation of a lot release testing policy should be considered by the NCL only if the prerequisites noted in section 5.2.2 have been addressed. Testing under inappropriate conditions may generate inaccurate or misleading data and cause unnecessary delay or rejection of lots that meet specifications.

The decision whether to conduct independent testing at the NCL should take into account the capacity of the NCL and the information available from other NRAs/NCLs that may also release the same product.

A testing policy should be established for each product and should consider four main questions:

1. Should the vaccine be tested by an independent authority?
2. If testing is required, what critical parameters should be tested by the NCL?
3. Should testing be done on every lot or on a reduced percentage of lots?
4. Are testing results available from another NCL?

Information influencing the decisions includes the nature of the final product (live or inactivated), the biological nature and complexity of source material, the complexity, robustness and level of control of the manufacturing process, and the nature and complexity of the quality control methods. An important factor is the manufacturer’s production history, which can be obtained from the summary protocol and/or yearly biological product reports, which, in some circumstances (see below), contain production and testing information. Other information to be considered includes the GMP inspection report, adverse events following immunization (AEFI) report, product complaints and other PMS safety and quality information. The testing policy for the same product at other NCLs may also be taken into consideration in establishing the testing policy.

A risk-based analysis for a particular product can help to determine if testing is required and, if so, at what frequency. A model procedure for such a risk analysis is given in Appendix 1.

An annual review of the important parameters, based on data provided in the lot release protocol to the NRA/NCL, can be used to support the evaluation of consistency for each product. Other information based on marketing authorization or inspection issues is also relevant but is not always available to the NCL, particularly when the NCL and the NRA are separate institutions or
when intergovernmental mutual recognition agreements for GMP inspections are not in place for imported products.

In some countries, yearly biological product reports are requested from the manufacturer for each vaccine (12). This information is used to assess product consistency. It is particularly helpful in markets where a limited number of lots is released, as it provides more comprehensive information on which to base the decision on whether to test, or the testing frequency and the type of testing required for each vaccine.

5.2.4 Criteria for selection of tests for lot release and percentage of lots to be tested

Once the decision to perform testing is taken, the NCL should concentrate on the selection of critical elements from the marketing authorization requirements to be tested, and the percentage of lots to be tested.

Key elements of focus where tests may be considered necessary include appearance, identity, potency, specific safety and, for some products, thermostability (e.g. OPV). Systematic testing of simple physical-chemical parameters may not be the highest priority when considering the best use of resources. Some parameters are better monitored through other tools, such as GMP compliance (e.g. sterility testing by aseptic process validation and environmental monitoring by the manufacturer). In all cases, the added value of the independent results for the tests chosen should be carefully considered in the context of the overall evaluation of the lot.

Testing is generally focused on the final product. The formulated final bulk may be tested in some cases (e.g. in the case of combination vaccines). Nevertheless, a complete evaluation of the properties in question may require assessment of upstream components (e.g. monovalent bulks). This may also be necessary if test procedures cannot be applied to final products (e.g. if the presence of adjuvant in the final product prevents immunochemical analyses).

Specific attention should be paid to new vaccines (as well as to new manufacturers) for which there is little accumulated experience, and to sophisticated combined vaccines for which testing and interpretation of results may be complicated.

The development and adoption of more effective test methods should be encouraged and should be approved by the NRA. If a different test method is used by the NCL, then – in case of data discrepancies between the manufacturer and the NCL – the approved test method defined in the marketing authorization should be used to solve the issue.

There should be a regular review of the testing policy, in order to re-evaluate the need and appropriateness in the current situation. Additional tests may be included, or existing tests deleted, as required. Informal testing outside
a planned programme and without sufficient preparation should be avoided, as this can generate non-relevant or misleading test results.

The percentage of lots of a given product to be covered by the testing programme should be clearly defined in advance. If a reduced percentage of lots is tested, the lots should be representative of the total production (e.g. selected number of bulks covering a maximum of final lots, or selection of filled lots issued from the same bulk). If less than 100% of lots are tested, the choice of lots to be tested should be in the hands of the NCL, and the manufacturer should not be aware in advance of which lots will undergo testing.

The percentage of lots tested should be monitored and revised, if necessary, on the basis of experience with the product and data from the yearly biological product report (e.g. good consistency over a significant period may lead to reduction of the percentage of lots covered, while observance of an undue number of failing results and/or specific testing issues may result in an increase in the percentage of lots to be tested).

Development of testing methodology and capability should begin as soon as possible for both the responsible NRA/NCL and the manufacturer, possibly at the stage of clinical trials. However, while testing of samples by an NCL for clinical trial approval is recommended in WHO guidelines (13), this is not considered lot release per se. Although additional guidance in this area is needed, this document focuses only on the lot release procedure for licensed products.

5.2.5 Importance of reference preparations for lot release

Appropriate use of reference preparations in independent testing is of critical importance for the interpretation of the results. This has a particular impact on the ability to make relevant comparisons between test results from different laboratories (e.g. manufacturer and NCL) and on the decision-making process.

Control charts of critical parameters of reference preparations should be kept, to monitor performance over time. This allows an overview of the performance of both the reference preparation and the method. For example, it could show if there has been a trend or a shift in the reference standard attributes – such as slope, intercept or 50% end-point – that may indicate problems with the stability of the reference standard or changes in other assay systems (e.g. animals, cells, critical reagents). Other examples of the utility of trend analysis are assay validity criteria based on 95% confidence intervals. If the assay validity criteria on any attribute of reference standard, slope, intercept or potency of control are based on 95% confidence intervals, and the actual data do not show approximately 95% acceptance of the assay based on that particular attribute, there may be problems with setting the limits or performance of that attribute.

The observations from this exercise can be important for feedback to marketing authorization authorities and/or bodies involved in biological
standardization activities, and can also be used to evaluate the appropriateness of the reference materials used and/or the need for new ones.

Reference Reagents are developed to improve standardization of assays. They are becoming increasingly important in the context of new vaccines, such as multicomponent vaccines. In many cases, the Reference Reagents are established and prepared by the manufacturer as they are often product specific. These Reference Reagents should be calibrated in International Units, against an International Standard where one exists.

5.2.6 Standards
The intention of the WHO International Standards is to serve as a basis for calibration of secondary standards (e.g. regional and national standards) (14). Generally, the International Standards are not used directly in the assays as a working standard. The regional or national standard is calibrated against the International Standard, to make a common working standard available to NCLs and manufacturers.

The regional or national standards should be established by a collaborative study, which should include the manufacturers. Practical aspects of secondary standard preparation need to be considered at regional level, and a suitable concept for development, establishment, distribution and use of regional reference preparations should be put in place.

5.2.7 Practical considerations
The number of samples of the final lot or upstream components requested by NCLs should be appropriate for the testing required, and the sampling procedures should ensure the representativeness of the lot in question. A system should be in place for recording, tracking and appropriate storage of all samples upon receipt from the manufacturer.

It may be necessary to obtain product-specific reference materials or reagents from the manufacturer. The amount requested should be relevant to the amount of testing to be performed and should not place undue stress on the supply of the materials, as stocks of these are often limited.

The time required for testing is an important issue, as it can greatly influence the supply chain and can have a significant impact when products have short shelf-lives. This can be of particular concern when in vivo tests, which can take several weeks to complete, are involved. Under certain circumstances, the NRA/NCL may agree to receive samples from manufacturers before they have completed their own test procedures, so that testing by the NCL is done in parallel. In such cases, the lot cannot be released by the NCL until all the test results from the manufacturer have been received (including the completed and signed final summary protocol with their test results). The NCL should evaluate
the risk and benefit of parallel testing, taking into account the frequency of rejection of lots by either the manufacturer or the NCL.

When animals are used for testing, the NCL should be aware of the potential variability of the source, housing and handling of animals. It is desirable to apply the “3R” principles (reduction, replacement, refinement) to minimize the use of animals, for ethical reasons. Validated in vitro alternatives should be favoured wherever possible. However, the type of testing should be driven by the scientific need for valid relevant data. Moreover, in the spirit of minimizing animal testing worldwide, agreements should be sought with the NCL of the exporting country or with other NCLs, in a mutual recognition or collaborative agreement, in order to utilize the results of animal testing already performed by another NCL.

5.2.8 Release specifications
NRA/NCL lot release should pertain only to products that have a valid marketing authorization in which specifications have been approved by the competent NRA of the country using the vaccine.

Since these specifications are used to judge the test results, it is important to have a mechanism in place to allow the testing NCL to be aware of the latest version of the approved licence specifications. Ideally, the responsible NCL staff should be involved in assessing the test methods, validity criteria and product specifications in the decision-making process for marketing authorization.

5.2.9 Evaluation of NCL results
The NCL test results should be assessed against the specifications approved in the marketing authorization dossier. It is understood that the variability expected in the results for a given test method for a given product should already be taken into account in the specifications. To be in compliance with the marketing authorization, the test results should fall within the defined acceptance criteria, which are based on the validated methodology used by the NCL, and the specifications approved in the marketing authorization (15).

The NCL should clearly define its retest policy and determine how, if applicable, the combination of results is carried out and how these results are evaluated. The acceptance criteria should also be predefined and laid down in relevant SOPs.

The NCL should have a predefined standard procedure for dealing with results that do not comply with the specifications. This should include confirmation that the results reflect the actual quality of the lot tested and are not due to analytical error by the NCL, or to the influence of variables unrelated to the product.

The manufacturer should be notified when an OOS result is confirmed and exchanges should ensue to try to identify the cause of the discrepancy.
A test report, including the results and outcome of all of the testing, should be prepared for final evaluation of the lot and the decision-making process.

A feedback mechanism from the NCL to the NRA and/or the GMP inspectorate is highly advisable, in order to coordinate and optimize regulatory actions (e.g. urging licence variation or refinement of product specification based on trend analysis).

6. Data monitoring

All critical quantitative data from quality-control results, and especially potency, from the manufacturer or other sources, should be used for trend analysis as an essential part of lot release. Statistical analysis should be conducted once sufficient data have been accumulated. The alert or warning limits and action limits of consistency trends should be defined on statistical grounds. In general, when data are distributed normally, ±2 and ±3 standard deviations of the mean are set for the alert or warning limits and action limits respectively. The variability and precision of the test should be considered when defining the limits. Care should be taken in interpreting such limits when they are based on small datasets. Trend analysis of key parameters may be requested from manufacturers or from the responsible NRA/NCL. More complex specific trend analysis statistical methods can be used when sufficient data and expertise are available, particularly when data are not normally distributed. In addition, a set of data from a certain period (e.g. 6 months or 1 year) should be analysed statistically, compared to data of the previous period, in order to detect any significant differences or shift in trends.

An SOP should be developed to describe this tracking and trending of manufacturers’ and, where available, the NCL’s results. This procedure will describe parameters to be tracked and trended, the frequency of periodic reviews, criteria for judgement, and actions to be taken in the case of outlier results, etc.

6.1 Trend analysis including data from the NCL

In cases where independent testing of lots is performed at the NCL, all data from these tests, including performance of reference standards and controls, should also be trended and analysed. It should be kept in mind that not all countries test all consecutive lots from a manufacturer. In such cases, the trends should be interpreted with caution and additional information from the manufacturer may be required, either directly or through contact with the relevant national inspectorate.

6.2 Comparison of results of the manufacturer with those of the NCL

Results from the NCL should be compared with those of the manufacturer. Any systematic differences should be documented. Any differences in trends should
be investigated and resolved, in collaboration with the manufacturer. Testing by
the NCL may, however, occur months after the manufacturer's release, so this
should be taken into consideration when the NCL makes the comparison.

7. Evaluation of the lot and the decision-making process

7.1 Establishment of decision-making procedures

The authority responsible for issuing a release certificate may differ between
countries. Therefore, it is critical that the roles and responsibilities of both the
NRA and the NCL are clearly defined, particularly when they are separate entities.
When all elements are available for final evaluation, a formal decision-making
process should be in place to decide whether the lot can be released. An SOP
should be in place to describe clearly the process and required elements for the
final decision. Good coordination and communication are needed, especially
when different bodies are involved in this process.

In order to provide continuity and to develop expertise on each product,
it is desirable that product specialists are assigned the responsibility for managing
the relevant information for particular products. A general lot release process
chart should be in place, outlining the lot approval process and the persons
responsible for each activity.

The competent authority’s approach to independent lot release should
be appropriately described in the NRA/NCL process charts. Procedures should
cover the options used: release upon review of summary protocol only and/or
release upon review of summary protocol plus independent testing by the NCL.
They should also define how and by whom the final decision is taken on the basis
of the formal written conclusions of the defined options used. SOPs or documents
are necessary to cover the essential elements presented below.

1. An SOP for summary protocol review should describe acceptance criteria
   for the completeness of the summary protocol, and all reviewing steps up
to and including the final conclusion on the summary protocol (e.g. need
for manufacturer’s correction, review of corrected pages, investigation,
conclusion).

   The NRA/NCL should produce a formal written conclusion regarding the
summary protocol review. A summary decision form should be filled out
to ensure compliance with approved specifications and should be signed by
the responsible staff.

2. An SOP should describe the acceptance criteria for NCL test results and
record all the individual test results in certificate(s) of analysis.
For the lot release following independent testing by the NRA/NCL, a formal written conclusion form containing the outcome of test results should be developed. A summary decision form should be used to capture the test results and ensure compliance with approved specifications, and should be signed by the responsible staff.

A retest policy should be developed in accordance with general quality assurance principles, in order to define the policy for retesting and handling of OOS results. In addition, an SOP should be in place to give guidance on retest policy according to product-specific recommendations (e.g. combination of results, calculation method). In the event of non-compliance, a full traceability investigation should be conducted on test reports and the manufacturer should be contacted for further investigation. As part of the quality assurance, in the event of derogation, an SOP should outline the decision-making process, including documentation and written criteria to support the decision made.

3. An SOP should be available that describes the acceptance criteria for release of vaccines in exceptional cases, which deviate from the normal procedure. Examples include release for an emergency/crisis situation, urgent need due to a critical supply shortage, when information is pending regarding correction of the summary protocol, or in the event of discrepancies between the test results of the NCL and the manufacturer. The procedure should be developed on the basis of a risk–benefit analysis that takes into account all available information. This should be applied only by the staff officially responsible for signing the release certificate. Documentation supporting compliance with approved specifications (summary protocol review and test reports, if applicable) should be included.

All steps in the decision-making process should be documented.

7.2 Recognition of, and confidence in, lot release by other NRAs/NCLs

In cases where a lot has already been released by another NRA/NCL, it may be possible to accept that lot for release on the basis of the existing release certificate. Processes for doing this may range from a list of countries that are acceptable to the importing country, through to mutual recognition agreements. Examples are described below.

Establishment of mutual recognition agreements is a legal approach. Many NRAs/NCLs use such agreements to: enhance international regulatory cooperation in order to maintain high standards of product safety and quality; reduce the regulatory burden for NRAs/NCLs and manufacturers; and improve the free flow of goods and increase the accessibility of medicinal products.
globally. Reciprocal mutual recognition of release certificates involves a number of legal aspects that should be addressed; however, the key to successful mutual recognition is the building of mutual confidence between the interested parties. This requires strong collaboration and communication between the different NRAs/NCLs and a good level of transparency.

Examples of agreements range from accepting the test results provided by another NCL, thus avoiding repeat testing and facilitating harmonization without compromising the safety and quality of the product, to full mutual recognition of the lot release certificate. The test results provided by another NCL can thus be used in addition to the protocol review by the local NRA/NCL, when they lot release the product.

Situations may exist where a two-way recognition of certificates or test results is not possible, owing to technical or other limitations. However, even in cases where reciprocity is not attainable, an NRA/NCL may still wish to recognize a release certificate from another NRA/NCL. This should be possible, provided the releasing NRA/NCL has clearly established procedures that are transparent and relevant to the NRA/NCL wishing to recognize the certificate or test results.

These types of approaches provide the advantage of limiting repeated evaluation and testing, and serve to streamline the release procedure.

It is important to note that the product manufacturers should be involved in the establishment of an agreement to share product information, since there are issues of confidentiality that need to be addressed.

When these types of arrangements are foreseen, specific SOPs should be developed to establish clearly what information is necessary and how it should be received and processed before final release on to the local market is accepted.

7.3 Release certificate issued by the NRA/NCL of a producing/releasing country for United Nations procurement

The responsible NRAs/NCLs are required to issue a certificate of release for vaccines that are distributed through United Nations agencies (16). Vaccines distributed through United Nations agencies are prequalified by WHO, to ensure that the products comply with the quality and safety standards established by the Organization. This release certificate is issued on the basis of, as a minimum, a review of the lot summary protocol for the relevant lot.

The responsible NRA/NCL plays a key role in ensuring that products meet the specifications outlined in the marketing authorization and WHO recommendations. This is achieved by maintaining regulatory oversight, assessing and approving changes to manufacturing processes – including testing and specifications, compliance with GMP – and PMS of AEFI. The release certificate issued by the responsible NRA/NCL should be forwarded by the United Nations agencies to the NRA/NCL of the receiving country, and the summary protocol will be provided upon request.
The receiving country may wish to review the summary protocol to develop its competency and have an overview of the vaccine quality.

In some countries, testing is undertaken on the product received by a competent laboratory, in order to strengthen the NCLs’ capacity and obtain information on the quality of the product at the receiving site. If a deficient result is detected, the responsible NRA/NCL should be consulted.

8. Lot release certificate

A release certificate for each vaccine lot should be issued by the NRA/NCL and sent to the manufacturer, confirming that the particular lot meets the approved specifications and related provisions. The release certificate is the official document authorizing the manufacturer to release the lot on to the market. The certificate may include the following information:

- name and address of the manufacturer;
- site(s) of manufacturing;
- trade name and/or common name of product;
- marketing authorization number;
- lot number(s) (including sub-lot numbers and packaging lot numbers if necessary);
- type of container;
- number of doses per container;
- number of containers/lot size;
- date of start of period of validity (e.g. manufacturing date) and/or expiry date;
- storage condition;
- signature and function of the authorized person and the agent authorized to issue the certificate;
- the date of issue of the certificate;
- the certificate number.

Other details – such as dosage form, strength of the product, registration code (NRA/NCL code for lot release) – may also be included in the certificate, according to the requirements of different countries.

The conclusion should be included clearly in the certificate, stating, for example: “The lot mentioned above complies with the relevant specification in
the marketing authorization and provisions for the release of biological products and has been approved for release”. The statement should also give an indication of the basis for the release decision (e.g. evaluation of the summary protocol, independent laboratory testing, specific procedures laid down in defined document, as appropriate).

For lots that fail to comply with the provisions, a different form should be issued, ideally with a different colour from the approval certificate, which clearly states that the lot is non-compliant.

It is advisable that the language on the lot release certificate is the national language, with an English translation of the information.

**Authors and acknowledgements**

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of Public Health, Brussels, Belgium; Mr A. Mhenni, National Agency for Sanitary and Environmental Control of Products, Tunis, Tunisia; Dr C. Milne, European Directorate for the Quality of Medicines & HealthCare, Strasbourg, France; Dr P. Minor, National Institute for Biological Standards and Control, Potters Bar, England; Dr T.O. Owolabi, Central Drug and Vaccine Laboratory, Lagos, Nigeria; Mr J. Peart, Health Canada, Ottawa, Canada; Dr M. Pfleiderer, Paul-Ehrlich-Institute, Langen, Germany; Dr D.M. Pascual, Centro para el Control Estatal de la Calidad de los Medicamentos, Havana, Cuba; Dr P. Richardson, European Medicines Agency, London, England; Dr A. Rinfret, Health Canada, Ottawa, Canada; Dr A. Sabouraud, Sanofi Pasteur, Marcy l’Et稍, France; Dr L. Set, Health Sciences Authority, Singapore, Singapore; Dr S. Siti Namtini, National Agency of Drug and Food Control, Jakarta, Indonesia; Mr J-M. Spieser, European Directorate for the Quality of Medicines & HealthCare, Strasbourg, France; Dr A. Sturgess, Merck & Co., Inc., Whitehouse Station, NJ, USA; Dr H. Rode, Health Canada, Ottawa, Canada; Dr D. Wood, World Health Organization, Geneva, Switzerland; and Ms L.M. Yong, Centre for Analytical Science, Health Sciences Authority, Singapore, Singapore.

The first draft of this Guideline was prepared by a drafting group whose members were: Dr I. Knezevic, World Health Organization, Geneva, Switzerland; Dr D. Lei, World Health Organization, Geneva, Switzerland; Dr F. Fuchs, Agence Francaise de Sécurité Sanitaire de Produits de Santé, Lyons, France; Dr M. Baca-Estrada, Health Canada, Ottawa, Canada; Dr C. Milne, European Directorate for the Quality of Medicines & HealthCare, Strasbourg, France; Mrs T. Jivapaisarnpong, Ministry of Public Health, Bangkok, Thailand; Dr R. Gupta, Food and Drug Administration, Rockville, MD, USA; Dr E. Chaves Leal, Instituto Nacional de Controle de Qualidade en Saúde, Rio de Janeiro, Brazil; Dr J. Wang, National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China; and Dr D. Xing, National Institute for Biological Standards and Control, Potters Bar, England.

The second draft of the Guideline was prepared by the drafting group at a meeting held in Cairo in March 2008. The third draft of the guideline was prepared by the group, taking into account comments on the second draft from national vaccine regulatory authorities and the vaccine industry.

The fourth draft was prepared Dr M. Baca-Estrada, Health Canada, Ottawa, Canada; Dr R. Gupta, Food and Drug Administration, Rockville, MD, USA; Mrs T. Jivapaisarnpong, Ministry of Public Health, Bangkok, Thailand; Dr I. Knezevic, World Health Organization, Geneva, Switzerland; Dr D. Lei, World Health Organization, Geneva, Switzerland; Dr C. Milne, European Directorate for the Quality of Medicines & HealthCare, Strasbourg, France; and Dr D. Xing, National Institute for Biological Standards and Control, Potters Bar, England, following a WHO informal consultation held in Thailand in November 2008 with the following participants: Dr M. Baca-Estrada, Health Canada, Ottawa, Canada;
Mr L. Belgharbi, World Health Organization, Geneva, Switzerland; Dr E. Chaves Leal, Instituto Nacional de Controle de Qualidade en Saúde, Rio de Janeiro, Brazil; Mr W. Effiok, Central Drug and Vaccine Laboratory, and Secretary, National Vaccine Advisory Committee, Lagos, Nigeria; Dr S. Fakhrzadeh, Ministry of Health and Medical Education, Tehran, Islamic Republic of Iran; Dr S. Jadhav, Serum Institute of India Ltd., Pune, India; Mrs T. Jivapaisarnpong, Ministry of Public Health, Bangkok, Thailand; Dr D. Khokal, Therapeutic Health Products Regulation Group, Singapore, Singapore; Dr I. Knezevic, World Health Organization, Geneva, Switzerland; Dr H. Langar, WHO Regional Office for the Eastern Mediterranean, Cairo, Egypt; Dr D. Lei, World Health Organization, Geneva, Switzerland; Dr A. Maes, Scientific Institute of Public Health, Brussels, Belgium; Ms D. Messaoud, National Agency for Sanitary and Environmental Control of Products, Tunis, Tunisia; Dr P. Minor, National Institute for Biological Standards and Control, Potters Bar, England; Dr S. Morgeaux, Agence Française de Sécurité Sanitaire de Produits de Santé, Lyons, France; Dr S.S. Nantini, National Agency of Drug and Food Control, Jakarta, Indonesia; Dr D.M. Pascual, Centro para el Control Estatal de la Calidad de los Medicamentos, Havana, Cuba; Dr M. Pfleiderer, Paul-Ehrlich-Institute, Langen, Germany; Dr C. Ponsar, GlaxoSmithKline Biologicals, Rixensart, Belgium; Dr C. Rolls, Therapeutic Goods Administration, Canberra, Australia; Dr A. Sabouraud, Sanofi Pasteur, Marcy l’Etoile, France; Ms J. Tresnabudi, Bio Farma, West Java, Indonesia; Dr J. Wang, National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China; Dr D. Wood, World Health Organization, Geneva, Switzerland; Dr D. Xing, National Institute for Biological Standards and Control, Potters Bar, England.

The fifth draft was prepared by Dr D. Lei, World Health Organization, Geneva, Switzerland; Dr P. Minor, National Institute for Biological Standards and Control, Potters Bar, England; and Dr C. Milne, European Directorate for the Quality of Medicines & HealthCare, Strasbourg, France, on the basis of comments from national regulators, the vaccine industry and the general public.

The sixth draft was prepared by Dr D. Lei, World Health Organization, Geneva, Switzerland, and the drafting group, on the basis of comments from the Expert Committee on Biological Standardization and the participants in the Committee meeting of 2009 and comments from the public consultation.

References


Appendix 1

A model procedure to document the decision-making process in lot release

This Appendix is intended to assist NCLs in documentation of the information and the process used in the evaluation of specific issues in vaccine lot release. Examples include:

- release of vaccine lots in emergency situations such as a vaccine shortage due to a disease outbreak, natural disaster, manufacturing problems (e.g. OOS) or other unforeseen circumstances;
- periodic evaluation of the frequency of independent testing (to consider modification, suspension or continuation of the current strategy);
- periodic evaluation of tests performed for lot release of a particular product (to consider deletion, inclusion or modification of given tests).

Since each situation is specific, it is expected that modifications to the structure and content of this template may be required in order for it to be applicable to different issues.

1. Issue
Define the problem/issue to be analysed.

2. Purpose/objective
Outline the purpose and/or objectives of this analysis (for instance, to evaluate the consistency of production of a vaccine) and explore whether changes to the frequency of independent testing or elimination of a specific test are justified on the basis of the consistency of production.

3. Background
Give a brief history of the problem/issue and identify critical information.

4. Issue analysis
List all key components of the issue to be analysed, taking into account relevant information from the NCL/NRA and manufacturers. Justify the results/conclusions with regulatory and scientific data, including published and unpublished information.
5. Options analysis

- List all the options considered to address the issue/problem, including the status quo.
- List and discuss the positive and negative aspects of each option.
- Outline the proposed solution or accepted alternative and why it was selected.
- If relevant, discuss the benefits and costs of the proposed solution compared to the benefits and costs of the other solutions.

6. Considerations
Identify any additional relevant information. For instance, discuss with other NCLs that are responsible for releasing this vaccine in other countries, in order to share information regarding production and quality control of this vaccine.

7. Recommendations
Indicate what the recommendation is and who is responsible for its approval.

8. Implementation and evaluation plan
Show how the proposed changes will be implemented in terms of timing, organizational and personnel changes and resource allocation.
Indicate when and how the proposed changes will be evaluated and against what benchmarks.

9. References and attachments
Include any references, reports and relevant information used in the risk analysis, such as GMP inspection report, regulatory post-marketing unit report, quality-control product report from the NCL, and/or a summary of decisions regarding variations submitted for regulatory approval.

I approve the recommendation proposed in this analysis,

Dr [insert name]
Director of National Control Laboratory
Annex 3

Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks

Replacement of Annex 1 of WHO Technical Report Series, No. 878

Abbreviations

1. Introduction
2. Historical overview
3. Scope
4. Definitions
5. General considerations
   5.1 Types of animal cell substrates
   5.2 Potential risks and risk mitigation associated with biologicals produced in animal cell cultures

Part A. General recommendations applicable to all types of cell culture production
   A.1 Good manufacturing practices
   A.2 Principles of good cell culture practice
   A.3 Selection of source materials
   A.4 Certification of cell banks by the manufacturer
   A.5 Cryopreservation and cell banking

Part B. Recommendations for the characterization of cell banks of animal cell substrates
   B.1 General considerations
   B.2 Identity
   B.3 Stability
   B.4 Sterility
   B.5 Viability
   B.6 Growth characteristics
   B.7 Homogeneity
   B.8 Tumorigenicity
   B.9 Oncogenicity
   B.10 Cytogenetics
   B.11 Microbial agents
   B.12 Summary of tests for the evaluation and characterization of animal cell substrates
Recommendations published by WHO are intended to be scientific and advisory. Each of the following sections constitutes guidance for national regulatory authorities (NRAs) and for manufacturers of biological products. If an NRA so desires, these recommendations may be adopted as definitive national requirements, or modifications may be justified and made by the NRA. It is recommended that modifications to these recommendations be made only on condition that the modifications ensure that the biological product is at least as safe and efficacious as that prepared in accordance with the recommendations set out below. The parts of each section printed in small type are comments for additional guidance, intended for manufacturers and NRAs, which may benefit from these details.
Abbreviations

ALS antilymphocyte serum
ATCC American Type Culture Collection
ATG antithymocyte globulin
ATS antithymocyte serum
BAV5 bovine adenovirus 5
BCG bacille Calmette–Guérin vaccine
bp base pairs
BPIV3 bovine parainfluenza type 3 virus
BPV bovine parvovirus
BRSV bovine respiratory syncytial virus
BSE bovine spongiform encephalopathy
BTV bluetongue virus
BVDV bovine viral diarrhoea virus
CCL continuous cell line
CEF chick embryo fibroblasts
CHO Chinese hamster ovary
CJD Creutzfeldt–Jakob disease
CPE cytopathic effect
CTL cytotoxic T-lymphocyte
CWD chronic wasting disease
DCL diploid cell line
EBV Epstein–Barr virus
ECB extended cell bank
EFSA European Food Safety Authority
ELISA enzyme-linked immunosorbent assay
EMA European Medicines Agency
EOP end of production
EOPC end-of-production cell
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>EPIC-PCR</td>
<td>exon-primed intron crossing PCR</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration (USA)</td>
</tr>
<tr>
<td>FFI</td>
<td>fatal familial insomnia</td>
</tr>
<tr>
<td>GBR</td>
<td>geographical BSE-risk level</td>
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<tr>
<td>GMP</td>
<td>good manufacturing practices</td>
</tr>
<tr>
<td>GSS</td>
<td>Gerstmann–Sträussler–Scheinker syndrome</td>
</tr>
<tr>
<td>HCP</td>
<td>hamster cheek pouch</td>
</tr>
<tr>
<td>HDC</td>
<td>human diploid cell</td>
</tr>
<tr>
<td>HEK</td>
<td>human embryonic kidney</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>IBR</td>
<td>infectious bovine rhinotracheitis virus</td>
</tr>
<tr>
<td>ICH</td>
<td>International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use</td>
</tr>
<tr>
<td>IFA</td>
<td>immunofluorescence assays</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>LCMV</td>
<td>lymphocytic choriomeningitis virus</td>
</tr>
<tr>
<td>MAb</td>
<td>monoclonal antibodies</td>
</tr>
<tr>
<td>MCB</td>
<td>master cell bank</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin–Darby canine kidney</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>miRNA</td>
<td>microRNA</td>
</tr>
<tr>
<td>MPS</td>
<td>massively parallel sequencing</td>
</tr>
<tr>
<td>NAT</td>
<td>nucleic acid amplification technique</td>
</tr>
<tr>
<td>NCL</td>
<td>national control laboratory</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer (cell)</td>
</tr>
<tr>
<td>NOD</td>
<td>non-obese diabetic</td>
</tr>
<tr>
<td>NRA</td>
<td>national regulatory authority</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>OIE</td>
<td>World Organisation for Animal Health</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCC</td>
<td>primary cell culture</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDL</td>
<td>population doubling level</td>
</tr>
<tr>
<td>PERT</td>
<td>product-enhanced reverse transcriptase</td>
</tr>
<tr>
<td>PMCA</td>
<td>protein misfolding cyclic amplification</td>
</tr>
<tr>
<td>PrP</td>
<td>prion protein</td>
</tr>
<tr>
<td>RCB</td>
<td>reference cell bank</td>
</tr>
<tr>
<td>rcDNA</td>
<td>residual cellular DNA</td>
</tr>
<tr>
<td>rDNA</td>
<td>recombinant DNA</td>
</tr>
<tr>
<td>REO3</td>
<td>reovirus 3</td>
</tr>
<tr>
<td>RFLP</td>
<td>restriction fragment length polymorphism</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcriptase</td>
</tr>
<tr>
<td>SCID</td>
<td>severe combined immunodeficiency</td>
</tr>
<tr>
<td>SCL</td>
<td>stem cell line</td>
</tr>
<tr>
<td>SPF</td>
<td>specific-pathogen-free</td>
</tr>
<tr>
<td>STR</td>
<td>short tandem repeats</td>
</tr>
<tr>
<td>SV</td>
<td>simian virus</td>
</tr>
<tr>
<td>TCID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>median tissue culture infective dose</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>TPD&lt;sub&gt;50&lt;/sub&gt;</td>
<td>tumour-producing dose at the 50% end-point</td>
</tr>
<tr>
<td>TSE</td>
<td>transmissible spongiform encephalopathy</td>
</tr>
<tr>
<td>vCJD</td>
<td>variant CJD</td>
</tr>
<tr>
<td>VNTR</td>
<td>variable number of tandem repeats</td>
</tr>
<tr>
<td>VSV</td>
<td>vesicular stomatitis virus</td>
</tr>
<tr>
<td>WCB</td>
<td>working cell bank</td>
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1. Introduction

Cell substrates are cells used to manufacture biological products. It is well established that both cell substrates themselves and events linked to cell growth can affect the characteristics and safety of the resultant biological products. Therefore, a thorough understanding of the characteristics of the cell substrate is essential in order to identify points of concern and to develop a quality control system that addresses these points.

Recent advances in the use and quality control of new animal cell substrates – particularly continuous cell lines (CCLs) and insect cells – led to the conclusion that an update to the WHO requirements (Requirements for the use of animal cells as in vitro substrates for the production of biologicals) (1) should be prepared. In order to facilitate the resolution of regulatory/scientific issues related to the use of animal (including human) cell cultures as substrates for the production of biological products, WHO initiated this revision of its requirements on cell substrates by establishing a WHO Study Group on Cell Substrates. Animal cells refer to cells derived from organisms classified as within the animal kingdom. This document is the result of the Study Group’s work, which included a wide range of consultations with individuals and organizations with expertise in this area. After comments were received from this consultative process, and from invited reviewers, further revision of the draft recommendations was undertaken and presented to the WHO Expert Committee on Biological Standardization in 2010. During the development of this document, guidance on the topic issued by other relevant organizations was considered. An effort was made to make the recommendations compatible with existing guidance whenever possible.

These Recommendations provide guidance to national regulatory authorities (NRAs), national control laboratories (NCLs) and manufacturers on basic principles and, in some cases, on detailed procedures that it is appropriate to consider in the characterization of animal cells proposed for use in the manufacture of biological products. Although the decision-making authority lies with the NRA, it is advisable that NCL experts on this topic should be consulted.

2. Historical overview

Historically, the major concerns regarding the safety of biological medicinal products manufactured in animal cells have been related to the possible presence of microbial contaminants and, in some cases, to the properties and components of the cells themselves – such as DNA and proteins.

For instance, in 1954 an experimental adenovirus vaccine was being developed and human tumour cells (HeLa) were rejected as the cell substrate in favour of “normal” cells (2). At that time, relatively little was known about the
biological mechanism(s) that lead to human cancer, so the risks to the recipients of a vaccine based on HeLa cells could not be assessed and quantified scientifically. Although “normal” cells were not defined, that decision led to the use of primary cell cultures (PCCs) from animals such as monkeys, hamsters and embryonated eggs for vaccine research and development (3).

The first Requirements for cell substrates were published by WHO in 1959 and related to the production of inactivated poliomyelitis vaccine in PCCs derived from the kidneys of clinically healthy monkeys (4). Those Requirements were revised and published in 1966 (5). Subsequently, other PCCs were used for the production of other viral vaccines.

In the 1960s, human diploid cells (HDCs) were developed and proposed as an alternative to primary monkey kidney cell cultures for production of polio virus vaccine, as well as for production of other viral vaccines. The rationale for using HDCs was based on the ability to:

- cryogenically preserve the cells at low population doubling levels (PDLs);
- establish and characterize cryopreserved banks of cells that later could be expanded to provide a standardized source of cells for many decades;
- extensively test recovered cells before use in vaccine production;
- demonstrate that the cells were free from detectable adventitious agents and that they were unable to form tumours when inoculated into immunosuppressed animals.

Thus, HDCs were normal by all of the then existing criteria. It was argued that because HDCs were normal and could be standardized, tested and used for many years, they were a significant improvement over PCCs.

The path to acceptance of HDCs was long and difficult, primarily because some members of the scientific community believed that HDCs might contain a latent and unknown human oncogenic agent and that such a theoretical agent posed a risk to the recipients of vaccines produced in HDCs. Numerous conferences and discussions of new data eventually led to the acceptance of HDCs as a substrate for viral vaccine production, and they continue to be used by many manufacturers for various viral vaccines that have a long history of safety and effectiveness. The concept of a master cell bank (MCB) and working cell bank (WCB) system and the characterization of the cell substrate were introduced during that period (6, 7).

Both the understanding of tumour cell biology and the technological tools that were available at that time were much more limited than they are today. As a result, the proponents of using HDCs for vaccine production based their
argument that the cells were normal, and therefore safe to use, on four points, namely: freedom from detectable adventitious agents; the finite life of HDCs; the diploid nature of HDCs; and the inability of HDCs to form tumours in various in vivo test systems.

In order to provide a high level of assurance that those four characteristics were stable, the initial lot release tests for each batch of a vaccine derived from HDCs included tests of the cell substrate for adventitious agents, karyology and tumorigenicity \((8, 9)\). The main question that was being addressed by the routine use of tumorigenicity tests was whether or not the production cell culture had undergone a contamination or transformation event such that it contained a mixture of “normal” and tumorigenic cells. It was eventually agreed that tumorigenicity testing was not sufficiently sensitive to detect a low level of tumorigenic cells, and that it was a waste of animals and time to carry out repeated testing of a cell line that had been well characterized and would be used in the context of a cell bank system. Thus, tumorigenicity tests were eventually required only for the characterization of an MCB (using cells at the proposed in vitro cell age for production or beyond) for both HDCs and CCLs \((10, 11)\).

In the 1970s, there was a need in clinical research for more interferon alpha (IFN-\(\alpha\)) than could be produced from primary human lymphocytes. In response, human tumour cells (Namalwa) grown in vitro were proposed as a cell substrate for the production of IFN-\(\alpha\). The primary concerns about the use of Namalwa cells were that they contained the Epstein–Barr virus (EBV) genome integrated into the cellular DNA, and that either whole virus or DNA containing viral elements could be transmitted to the recipients of the IFN-\(\alpha\) product. Nevertheless, by the end of the 1970s, regulatory agencies had allowed human clinical studies to commence, and the product was eventually approved in several countries. Among the most important factors contributing to those decisions was the fact that IFN, as opposed to live viral vaccines, was not a replicating agent, and IFN-\(\alpha\) was being used as a therapeutic product rather than a prophylactic one, thus representing different risk–benefit considerations. In addition, technology had advanced significantly so that IFN-\(\alpha\) could be highly purified and the purification process could be validated to demonstrate that EBV and cellular DNA were undetectable in the final product, within the limits of the assays then available, which permitted risk mitigation.

In the 1980s, advances in science and technology led to the development of recombinant DNA (rDNA)-derived proteins and monoclonal antibodies (MAbs). Animal cells with the capacity to grow continuously in vitro (CCLs) were the substrates of choice for those products because of the ease with which they could be transfected and engineered. Also, in contrast to PCCs and HDCs, CCLs grew rapidly to achieve a high density and expressed a variety of products at high concentrations. Chinese hamster ovary (CHO) cell lines became widely
used for rDNA products, and hybridomas of various types were required for the production of MAbs. The use of such cells as substrates in the manufacture of a large array of potentially important biological medicinal products raised safety concerns once again. A scientific consensus emerged from numerous conferences that there are three major elements of potential concern related to animal-cell substrates – DNA, viruses and transforming proteins. In 1986, WHO established a WHO Study Group on Cell Substrates to examine cell substrate issues in greater depth.

The Study Group concluded that there is no reason to exclude CCLs from consideration as substrates for the production of biologicals, and that CCLs are in general acceptable when the manufacturing process is shown to eliminate potential contaminating viruses that are pathogenic for humans and to reduce DNA to acceptable levels and/or eliminate its biological activity (12). The Study Group’s emphasis on infectious agents as the major risk factor was based largely on experience of virus transmission and disease occurring through contaminated biological products (e.g. hepatitis B virus and HIV in factor VIII). WHO’s Requirements for CCLs used for the production of biologicals were published in 1987 (13). On the basis of a review of more recent data, those Requirements were revised in 1998 to raise the acceptable level of rcDNA to 10 ng per parenteral dose. In addition, it was pointed out that beta-propiolactone, a viral inactivating agent, may also destroy the biological activity of DNA. Use of this agent therefore provides an additional level of confidence, even when the amount of DNA per dose may be substantial (1).

During the 1990s and into the 2000s, a variety of CCLs were explored as cell substrates for biological products in development because, like the cell lines referred to above, they offered significant advantages during production (e.g. rapid growth and high expression). They include the tumorigenic cell lines – HeLa for adeno-associated virus vectored HIV vaccines, PER.C6 for influenza and HIV vaccines, Madin–Darby canine kidney (MDCK) for influenza vaccines, and 293ORF6 for HIV vaccines. More recently, insect cell lines and stem cell lines (SCLs) have been proposed for the manufacture of biological products, and such cells introduce a new set of challenges with regard to their evaluation and characterization.

The acceptability of a given cell type (primary, diploid, stem or continuous) as a substrate for the production of a specific biological product depends on a variety of factors, including in-depth knowledge of the cell type’s basic biological characteristics. It is important to recognize that the tumorigenic potential of a CCL is only one of many factors, including the extent to which the manufacturing process reduces or eliminates cellular factors that may be of concern, to be considered. An assessment of the totality of the data available is needed in order to determine whether a product manufactured in a given cell substrate is potentially approvable.
The following Recommendations provide guidance to manufacturers, NRAs and NCLs on the evaluation of animal cell cultures used as substrates for the production of biological medicinal products, and for the characterization of cell banks.

The main changes compared to the Requirements published in WHO Technical Report Series, No. 878, Annex 1, include the following:

- general manufacturing recommendations applicable to all types of cell culture production have been updated;
- some considerations for evaluating new cell substrates such as insect cells and stem cells have been added;
- definitions have been updated and expanded in number and scope, and are moved to an earlier point in the document;
- the structure of the document has been modified to include more background information, and the applicability of various sections to different types of cell substrates is highlighted;
- a new section on risk-reduction strategies during the manufacture of biological products has been added;
- a section on good cell culture practice has been added;
- the section on selection of source materials has been updated, and the detailed methods used to test for bovine viruses in serum have been added in Appendix 1;
- tumorigenicity testing has been updated, and a model protocol for the nude mouse model has been added in Appendix 2;
- oncogenicity testing of tumorigenic cell lysates has been added, and a model protocol is added in Appendix 3;
- recommendations for acceptable levels of residual cellular DNA are product specific and are not specifically addressed;
- recommendations for microbial agents testing have been updated.

3. Scope

These Recommendations supersede previous WHO Requirements or Recommendations describing procedures for the use of animal cell substrates for the production of biological medicinal products (1, 13).

Some of the recommendations may also be useful in the quality control of specific biological products during the manufacturing process, but it is beyond the scope of this document to recommend quality control release tests. Likewise, risk-based assessments related to product approvals are beyond the scope of this
document. Requirements or recommendations for individual products should be consulted for such assessments.

Cells modified by recombinant DNA technology have been increasingly used in the manufacture of novel medicinal products, and specific considerations for those products are addressed elsewhere (1, 10, 14, 15). Nevertheless, a number of generic issues apply to genetically modified and other cell substrates.

These Recommendations specifically exclude all products manufactured in embryonated eggs, microbial cells (i.e. bacteria and yeast) and plant cells. Also excluded are whole, viable animal cells such as stem cells when they are used directly for therapy by transplantation into patients or when they are developed into SCLs for the purpose of using them as therapeutic agents by transplantation. In those cases, characterization tests should be discussed with the NRA/NCL. Nevertheless, SCLs used for the production of biological products such as growth factors and vaccines should comply with these recommendations.

Some of the general recommendations given here (see sections A.1 to A.5) are applicable to all animal cell substrates. More specific guidance for PCCs can be found in the relevant documents published by WHO (e.g. production of poliomyelitis vaccine in primary monkey kidney cells) (4, 5).

Cell substrates should be developed and used in accordance with applicable requirements of the NRA/NCL.

In general, it is not consistent with good manufacturing practices (GMP) to retest materials that have already been released for further manufacture, so justification would be necessary before such retesting is undertaken. Thus, the scope of this document is intended to cover cell substrates as new cell banks are established. The specific circumstances under which the retesting of already established and released cell banks would be appropriate should be discussed with the responsible NRA/NCL.

Recommendations published by WHO are intended to be scientific and advisory in nature. The parts of each section printed in normal type have been written in the form of recommendations so that, should an NRA/NCL so desire, this text may be adopted as it stands as the basis of national or regional requirements. The parts of each section printed in small type are comments or additional points that may be considered in some cases.

4. Definitions

The definitions given below apply to the terms used in these recommendations. The terms may have different meanings in other contexts.

Adventitious agent: contaminating microorganisms of the cell culture or source materials including bacteria, fungi, mycoplasmas/spiroplasmas, mycobacteria, Rickettsia, protozoa, parasites, transmissible spongiform
encephalopathy (TSE) agents, and viruses that have been unintentionally introduced into the manufacturing process of a biological product.

The source of these contaminants may be the legacy of the cell line, the raw materials used in the culture medium to propagate the cells (in banking, in production, or in their legacy), the environment, personnel, equipment or elsewhere.

**Biological medicinal product**: biological medicinal product is a synonym for biological product or biological described in the WHO Technical Report Series. The definition of a medicinal substance used in treatment, prevention or diagnosis as a “biological” has been variously based on criteria related to its source, its amenability to characterization by physicochemical means alone, the requirement for biological assays, or arbitrary systems of classification applied by regulatory authorities. For the purposes of WHO, including the current document, the list of substances considered to be biologicals is derived from their earlier definition as “substances which cannot be fully characterized by physicochemical means alone, and which therefore require the use of some form of bioassay” (16). However, developments in the utility and applicability of physicochemical analytical methods, improved control of biological and biotechnology-based production methods, and an increased applicability of chemical synthesis to larger molecules have made it effectively impossible to base a definition of a biological on any single criterion related to methods of analysis, source or method of production. Nevertheless, many biologicals are produced using in vitro culture systems.

Developers of such medicinal products that do not fit the definition of biological medicinal product provided in this document should consult the relevant NRAs for product classification and the licensing application pathway.

**Biotherapeutic**: for the purpose of this document, a biotherapeutic is a biological medicinal product with the indication of treating human diseases.

**Cell bank**: a collection of appropriate containers whose contents are of uniform composition, stored under defined conditions. Each container represents an aliquot of a single pool of cells.

The individual containers (e.g. ampoules, vials) should be representative of the pool of cells from which they are taken and should be frozen on the same day by following the same procedure and by using the same equipment and reagents.

**Cell culture**: the process by which cells are grown in vitro under defined and controlled conditions, where the cells are no longer organized into tissues.
**Cell line:** type of cell population with defined characteristics that originates by serial subculture of a primary cell population that can be banked.

Cloning and subcloning steps may be used to generate a cell line. The term “cell line” implies that cultures from it consist of lineages of some of the cells originally present in the primary culture.

**Cell seed:** a quantity of well-characterized cells that are frozen and stored under defined conditions, such as in the vapour or liquid phase of liquid nitrogen, in aliquots of uniform composition derived from a single tissue or cell, one or more of which would be used for the production of a master cell bank. Cell seed is also referred to as a pre-MCB or seed stock. It may be made under conditions of GMP or under the manufacturer’s research and development conditions.

**Cell substrate:** cells used to manufacture a biological product.

The cells may be primary or cell lines, and may be grown in monolayer or suspension culture conditions. Examples of cell substrates include primary monkey kidney, MRC-5, CHO, and Vero cells.

Cells used to generate essential components of a final product (e.g. Vero cells for the generation of “reverse genetics” virus for use in seeding vaccine production) are considered to be “preproduction” cell substrates. Cells used to manufacture the bulk product (e.g. packaging cell lines for gene therapy vectors, Vero cells for vaccine production, or CHO cells for recombinant protein expression) are considered to be “production” cell substrates.

**Continuous cell line (CCL):** a cell line with an apparently unlimited capacity for population doubling. It is often referred to as “immortal” and was in the past referred to as “established”.

**Diploid cell line (DCL):** a cell line with a finite in vitro lifespan in which the chromosomes are paired (euploid) and are structurally identical to those of the species from which they were derived.

While this definition is accurate for standard chromosome preparations, a given human diploid cell line may contain genetic variations that will be reflected in a Giemsa-banding pattern that differs from the standard. Gene expression differences may also be found.

This definition is based on experience and the current understanding of the in vitro behaviour of human cells that are not of stem cell origin.

**DNA infectivity:** the capacity of cellular DNA to generate an infectious virus following introduction of that DNA into appropriate cells. The viral genome may be integrated or extrachromosomal.
**Endogenous virus:** a virus whose genome is present in an integrated form in a cell substrate. Endogenous viruses are present in the genome of the original animal from which the cells were derived. They may or may not encode an intact or infectious virus.

**End-of-production cells (EOPCs):** cells harvested at or beyond the end of a production (EOP) run.

In some cases, production cells are expanded under pilot-plant scale or commercial-scale conditions.

**Extended cell bank (ECB):** cells cultured from the MCB or WCB and propagated to the proposed in vitro cell age used for production or beyond.

**Functional integrity:** the culture sustains the expected performance related to its intended use under specified conditions (e.g. expression of secreted product at a consistent level, production of expected yield of virus).

**Immortalized:** having an apparently unlimited capacity for population doubling.

**Indicator cells:** cells of various species used in the in vitro adventitious agent test that are intended to amplify adventitious viruses to promote their detection. Generally, this would include a human diploid cell line (such as MRC-5), a monkey kidney cell line (such as Vero cells) and a cell line of the same species and tissue as the cell bank. The purpose of these cell lines is to “indicate” a viral infection of the cell bank either through observation of cytopathic effect (CPE) during and after an appropriate observation period or by haemadsorption and/or haemagglutination at the end of the observation period. Thus they are referred to as “indicator” cells. The cell bank may be analysed on such indicator cells either by co-cultivation or by passage of cell lysates or spent culture supernatants from the cell bank on to the indicator cells.

**In vitro cell age:** the measure of time between the thaw of the MCB vial(s) to the harvest of the production vessel, measured by elapsed chronological time, by population doubling level of the cells, or by passage level of the cells when subcultivated by a defined procedure for dilution of the culture.

**Latent virus:** a virus is considered to be latent when the viral genome is present in the cell without evidence of active replication but with the potential to be reactivated.

**Master cell bank:** a quantity of well-characterized cells of animal or other origin, derived from a cell seed at a specific PDL or passage level, dispensed into multiple containers, cryopreserved and stored frozen under defined conditions, such as the vapour or liquid phase of liquid nitrogen in aliquots of uniform composition. The MCB is prepared from a single homogeneously mixed pool of cells. In some cases, such as genetically engineered cells, the MCB may be prepared from a selected cell clone established under defined conditions.
Frequently, however, the MCB is not clonal. It is considered best practice for the MCB to be used to derive working cell banks.

**Oncogenicity:** the capacity of an acellular agent – such as a chemical, virus, viral nucleic acid, viral gene(s) or subcellular element(s) – to cause normal cells of an animal to form tumours.

Oncogenicity is distinct from tumorigenicity (see “Tumorigenicity”). The tumours that arise in an oncogenicity test are of host origin, whereas in a tumorigenicity test, the tumours are derived from the inoculated cells.

**Parental cells:** cells that are manipulated to give rise to a cell substrate. For hybridomas, it is usual also to describe the parental cells as the cells to be fused.

Manipulation may be as simple as the expansion of a primary cell culture to provide early-passage cells, or it may be a more complex activity such as developing a hybridoma or transfected clone. Both processes would provide a cell seed. The parental cells may refer to any stage prior to the preparation of the cell seed. Examples of a parental cell are WI-38 and MRC-5 at very early passage, Vero at passage 121, and CHO before the introduction of a DNA construct to produce a recombinant cell. In certain situations (e.g. myeloma cells), there may be a lineage of identified stable parental clones; thus, the term “parental cell” would normally refer to the cells used immediately prior to generation of the cell seed.

**Passage:** the process of transferring of cells, with or without dilution, from one culture vessel to another in order to propagate them, and which is repeated to provide sufficient cells for the production process.

This term is synonymous with “subculture”. Cultures of the same cell line with the same number of passages in different laboratories are not necessarily equivalent because of differences in cell culture media, split ratios, and other variables that may affect the cells. This is a more important consideration for SCLs and CCLs than for DCLs. Population doubling is the preferred method of estimating cell-line age and, whenever possible, should be used instead of passage. However, it also may be appropriate to quantify culture duration of CCLs by the number of subcultivations at a defined seeding density at each passage or time in days.

**Population doubling:** a twofold increase in cell number.

**Population doubling level:** the total number of population doublings of a cell line or strain since its initiation in vitro. A formula to use for the calculation of population doublings in a single passage is:

\[
\text{number of population doublings} = \log_{10} \left( \frac{N}{N_0} \right) \times 3.33
\]
where $N =$ number of cells in the growth vessel at the end of a period of growth and $N_0 =$ number of cells plated in the growth vessel (17). It is best to use the number of viable cells or number of attached cells for this determination.

**Primary culture:** a culture started from cells, tissues or organs taken directly from one or more organisms. A primary culture may be regarded as such until it is successfully subcultured for the first time. It then becomes a cell line if it can continue to be subcultured at least several times.

**Production cell cultures:** A collection of cell cultures used for biological production that have been prepared together from one or more containers from the WCB or, in the case of PCCs, from the tissues of one or more animals.

**Residual cellular DNA (rcDNA):** cell substrate DNA present in the final product.

**Specific-pathogen-free (SPF):** animals known to be free of specific pathogenic microorganisms and reared in an environment that maintains that state. SPF animals are usually raised in biosecure facilities and their health status is monitored on an ongoing basis. The SPF status simply provides an assurance that the stock is not infected with the specified pathogens. SPF animals are not disease free, nor are they disease resistant; they may carry pathogens other than those from which they are specified to be free.

**Stem cell line:** a continuous cell line generated from stem cells rather than from normal or diseased differentiated tissue.

**Transmissible spongiform encephalopathy:** the transmissible spongiform encephalopathies (TSEs) are a group of fatal neurodegenerative diseases which include classical and variant Creutzfeldt–Jakob disease (CJD), Gerstmann–Sträussler–Scheinker syndrome (GSS), fatal familial insomnia (FFI) and Kuru in humans, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in mule, deer and elk, and scrapie in sheep and goats.

**Tumorigenicity:** the capacity of a cell population inoculated into an animal model to produce a tumour by proliferation at the site of inoculation and/or at a distant site by metastasis.

Tumorigenicity is distinct from oncogenicity (see “Oncogenicity”).

**WHO reference cell bank (RCB):** a cryopreserved stock of cells prepared from a single homogeneous pool of cells prepared under defined conditions and subjected to characterization tests. The purpose of such a bank is to serve as a well-characterized cell seed for the preparation of MCBs that will be extensively characterized by manufacturers and that have a high probability of meeting these recommendations.

**Working cell bank:** a quantity of well-characterized cells of animal or other origin, derived from the MCB at a specific PDL or passage level, dispensed into multiple containers, cryopreserved and stored frozen under defined
conditions, such as in the vapour or liquid phase of liquid nitrogen, in aliquots of uniform composition. The WCB is prepared from a single homogeneously mixed pool of cells. One or more of the WCB containers is used for each production culture.

5. General considerations

5.1 Types of animal cell substrates

5.1.1 Primary cell cultures (PCCs)

PCCs have played a prominent role in the development of biology, and of virology in particular. Cultures of PCCs from different sources have been in worldwide use for the production of live and inactivated viral vaccines for human use for many decades. For example, PCCs of monkey kidney cells have been used for the production of inactivated and oral poliomyelitis vaccines since the 1950s.

Major successes in the control of viral diseases, such as poliomyelitis, measles, mumps and rubella, were made possible through the wide use of vaccines prepared in PCCs, including those from chicken embryos and the kidneys of monkeys, dogs, rabbits and hamsters, as well as other tissues.

PCCs are viable cells of disaggregated tissues that are initiated as in vitro cell cultures, usually as adherent cells. Many cell types are present and a primary culture can be a complex mixture of cells that may be influenced by the process and conditions under which they were harvested, disaggregated and introduced to in vitro culture. Not all cells in a primary culture will have the capacity to replicate. Particular care should be given to establishing highly reproducible procedures for tissue disaggregation, cell processing and culture initiation, as well as reproducible culture conditions and nutrition.

PCCs obtained from wild animals usually show a high frequency of viral contamination. For instance, monkey kidney cell cultures may be contaminated with one or more adventitious agents, including simian viruses.

If PCCs are necessary for the production of a given biological, the frequency of contaminated cell cultures can be significantly reduced by screening the source animals carefully for the absence of such viruses. Viruses can be detected by molecular tests such as polymerase chain reaction (PCR), and by looking for the presence of circulating antibodies to those viruses in the source animals. The use of animals bred in a carefully controlled colony, especially those that are SPF, is strongly recommended. Nevertheless, as suitable alternative cell substrates become available, PCCs are less likely to be used in the future. WHO has promoted the replacement of animals for experimental purposes, both for ethical reasons (18) and in the interests of progressive improvement in product safety and quality.
5.1.1 Advantages of PCCs

- PCCs are comparatively easy to prepare using simple media and bovine serum.
- They generally possess a broad sensitivity to a variety of viruses, some of which are cytopathic.

5.1.2 Disadvantages of PCCs

- Contamination by infectious agents is a higher risk than with DCLs and CCLs.
- The quality and viral sensitivity of cultures obtained from different animals are variable.
- Although cell cultures derived from non-human primates have been widely used in the past, it has become increasingly difficult to obtain and justify the use of such animals for this purpose.
- PCCs cannot be tested as extensively as DCLs or CCLs.

5.1.2 Diploid cell lines (DCLs)

The practicality of using human DCLs for the production of viral vaccines was demonstrated in the 1960s. Experience gained with oral poliomyelitis and other viral vaccines in successfully immunizing billions of children in many countries has shown clearly that such substrates can be used in the production of safe and effective vaccines (3).

The essential features of DCLs of human (e.g. WI-38, MRC-5) and monkey (i.e. FRhL-2) origin are:

- they are cells passaged from primary cultures that have become established as cell lines with apparently stable characteristics over numerous PDLs;
- they have a finite capacity for serial propagation which ends in senescence, a state in which the culture ceases to replicate; the cells remain alive and metabolically active but may show morphological and biochemical changes, some of which begin to appear before replication ceases;
- they are non-tumorigenic;
- they display diploid cytogenetic characteristics with a low frequency of chromosomal abnormalities of number and structure until they enter senescence.
Substantial experience has been accumulated on the cytogenetics of WI-38 and MRC-5 since the 1960s, and ranges of expected frequencies of chromosomal abnormalities have been published (19, 20). Similar data are available for FRhL-2 (21). More sophisticated cytogenetic techniques (e.g. high-resolution banding, comparative genome hybridization) (22, 23) have demonstrated the presence of subtle chromosomal abnormalities that were previously undetectable. Recent studies have shown that subpopulations of human DCLs with such abnormalities may appear and disappear over time, that they are non-tumorigenic and that they undergo senescence in the same manner as the dominant population. Thus, possessing a stable karyotype might not be such an important characteristic as was previously thought.

5.1.2.1 Advantages of DCLs

- DCLs can be well characterized and standardized.
- Production can be based on a cryopreserved cell bank system that allows for consistency and reproducibility of the reconstituted cell populations.
- A cell bank system usually consists of cell banks of defined population doubling or passage levels that generally include an MCB and a WCB.
- Unlike the CCLs and SCLs discussed below, DCLs are not tumorigenic and therefore do not raise the potential safety issues associated with CCLs and SCLs.

5.1.2.2 Disadvantages of DCLs

- DCLs are not easy to use in large-scale production, although they have been cultivated using bioreactor technology employing the microcarrier or multilayer method.
- In general, they have more fastidious nutritional requirements than other cell substrates.
- They may be difficult to adapt to serum-free growth.
- DCLs are more difficult to transfect and engineer than CCLs, and DCLs require immortalization before they can be engineered (e.g. they are not permissive for the production of vaccine vectors that require complementation, since they cannot be engineered readily to express complementing proteins).

5.1.3 Continuous cell lines (CCLs)

Some CCLs have been used for the production of safe and effective biotherapeutics and vaccines since the 1980s.
CCLs have the potential for an apparently indefinite in vitro lifespan and have been derived by the following methods:

- serial subcultivation of a PCC of a human or animal tumour (e.g. HeLa cells);
- transformation of a normal cell having a finite lifespan, with an oncogenic virus or viral sequence (e.g. B lymphocytes transformed by EBV or transfected with viral sequences such as in PER.C6);
- serial subcultivation of a primary cell population derived from normal tissue that generates a dominant cell population having an apparently indefinite lifespan, often described as spontaneous transformation (e.g. Vero, BHK-21, CHO, MDCK, Hi5);
- fusion between a myeloma cell and an antibody-producing B lymphocyte to produce a hybridoma cell line;
- use of ectopically expressed genes involved in the cell cycle, such as hTERT telomerase gene, to enable indefinite replication of normal human cells.

CCLs may display a consistent modal chromosome number (e.g. MDCK, Vero), and although the karyotype of individual cells in a culture at one point in time may vary, the range of chromosome numbers per cell will usually show characteristic limits. However, other CCLs, such as highly tumorigenic cells including HeLa, may show variation in modal number and a wider drift in the range of the number of chromosomes per cell.

In the early stage of establishing a cell line, significant diverse karyotypes and changes in karyotype may be observed. However, a characteristic chromosome component may emerge with continued passage, presumably as a dominant cell population develops.

5.1.3.1 Advantages of CCLs

- CCLs can be characterized extensively and their culture conditions can be standardized.
- Production of CCLs can be based on a cell bank system, which allows for consistency and reproducibility of the reconstituted cell populations for an indefinite period.
- CCLs generally grow more easily than DCLs, using standard media.
- Most CCLs can be adapted to grow in a serum-free medium.
- CCLs can usually be grown on microcarriers for large-scale production in bioreactors.
- Some can be adapted to grow in suspension cultures for large-scale production in bioreactors.
5.1.3.2 Disadvantages of CCLs

- CCLs may express endogenous viruses, and some are tumorigenic in immunosuppressed animal models.
- Theoretical risks identified by the 1986 Study Group (e.g. nucleic acids, transforming proteins and viruses) need to be taken into account.

5.1.4 Stem cell lines (SCLs)

Stem cells differ from other types of cells because they sustain a predominant stem cell population while simultaneously retaining the capacity to produce cell progenitors of differentiated cell types of almost all human tissues (i.e. they are pluripotent). Pluripotent SCLs have an apparent capacity to generate cell types of all three human germ layers and may be capable of generating in vitro models of any tissue in the human body. At the time these Recommendations were written (2010), two types of pluripotent SCLs – human embryonic stem cells and induced pluripotency stem cell lines – had been isolated and were considered to possibly have the capabilities to prove useful for manufacturing biologicals. The property of pluripotency is sustained through numerous cycles of cell division. SCLs may be derived from early-stage embryonic, fetal or adult tissues. Typically, specialized media and environmental conditions such as the attachment matrix are required for the growth of SCLs in vitro, in order for them to maintain the undifferentiated state. While most stem cell research and development has been directed towards transplantation of stem cells for therapeutic purposes, efforts also have been made to explore a variety of SCLs as cell substrates for the production of biologicals.

Key considerations for the culture and control of such cell lines have been developed (24). These include the fundamental issues common to the maintenance of all cell lines, but also include the need for appropriate ethical governance regarding donor consent and careful attention to periodic confirmation of phenotype, absence of non-diploid cells, and sustained pluripotent capacity.

It has recently been shown that conditioned medium from SCLs can have regenerative properties. Such preparations produced from human embryonic stem cells have shown regenerative capabilities, including repair of myocardial infarction in animal models (25). This raises the possibility of stem cells being used as a substrate to produce a variety of biologically active molecules. SCLs can, in some respects, be considered as diploid cells, but they do not appear to have the finite lifespan characteristic of human diploid fibroblast cultures. In human embryonic stem cell cultures, clonal variants with chromosomal abnormalities are known to arise. While a diploid and non-transformed nature is considered a prerequisite for cell therapy applications, transformed SCLs might be considered as a form of CCL for the manufacture of biologicals. Because they do not fall easily within any one category of substrate already discussed, SCLs are identified separately in this document.
5.1.4.1 Advantages of SCLs

- SCLs can be well characterized and their culture conditions standardized.
- Production can be based on a cell bank system, allowing consistency and reproducibility of the reconstituted cell populations for an indefinite period.
- Some SCLs may be adapted to grow in suspension cultures for large-scale production in bioreactors.
- SCLs may produce unique proteins of potential importance as biotherapeutics.
- SCLs have the potential to generate cells and tissue-like structures that may permit the expression of agents that are currently considered unculturable in vitro.

5.1.4.2 Disadvantages of SCLs

- Subculture techniques commonly used for SCLs are laborious.
- SCLs may produce growth proteins with undefined effects on adult cells/tissues.
- SCLs usually require complex media that may have a TSE risk.
- Rapid development of differentiated cells also means that they are difficult to control in vitro.
- There is little experience with the use of SCLs as a cell substrate to manufacture biological products.

5.2 Potential risks and risk mitigation associated with biologicals produced in animal cell cultures

The main potential risks associated with the use of biologicals produced in animal cells are directly related to contaminants from the cells. These risks fall into three categories, namely:

- viruses and other transmissible agents;
- cellular nucleic acids (DNA and RNA);
- growth-promoting proteins.

In addition, cell-derived inhibiting or toxic substances are theoretically possible. A summary of the risk assessment for each follows. More comprehensive information has been published elsewhere on the risks associated with contaminating DNA and growth-promoting proteins (26–35).
In 2010, NRAs and WHO were made aware of new information regarding the presence of DNA sequences of porcine circovirus in live-attenuated rotavirus vaccines. The detection of these sequences by the use of advanced analytical methods raised complex questions (e.g. the evaluation of the potential risk, specific testing of vaccines, and the general use of these methods for the characterization of vaccine cell substrates). The power of the new methodology that was used (i.e. massively parallel (deep) sequencing (MPS)) may reveal the presence of adventitious agents that might not be detected with current methods. While the implementation for routine use of such methods has benefits as well as challenges and risks, NRAs need to be prepared for similar situations. Consideration should be given to making a risk assessment and potentially introducing risk-mitigation strategies in such circumstances.

5.2.1 Viruses and other transmissible agents

There is a long history of concern regarding the potential transmission of viruses and other infectious agents that may be present in cell substrates. WHO reviewed this area in 1986 through a Study Group that pointed out that, as described below, cells differ with respect to their potential for carrying viral agents that are pathogenic for humans.

Primary monkey kidney cells have been used to produce billions of doses of poliomyelitis vaccines since they were first developed in the 1950s and, although viruses such as SV40 were discovered in rhesus monkey kidney cells, control measures were introduced to eliminate or reduce as much as possible the risk of viral contamination associated with the manufacture of vaccines in cells containing those viruses. Additional controls may be needed as new viral agents are identified and technologies to detect them are developed.

Human and non-human primate lymphocytes and macrophages may carry latent viruses such as herpesviruses and retroviruses. CCLs of non-haematogenous cells from human and non-human primates may contain viruses or have viral genes integrated into their DNA. In either case, virus expression may occur under conditions of in vitro culture.

Avian tissues and cells may harbour exogenous and endogenous retroviruses, but there is no evidence of transmission of disease to humans from products prepared using these substrates. For example, large quantities of yellow fever vaccines were produced for many years in eggs that contain avian leukosis viruses, but there is no evidence that these products have transmitted disease in their long history of use for human immunization. Nevertheless, the potential for transmitting avian retroviruses should be reduced as much as possible, through control measures during manufacture.

Rodents may harbour exogenous and endogenous retroviruses, lymphocytic choriomeningitis virus, and hantaviruses, and a range of other
potentially zoonotic viruses. While contamination with the rodent viruses in the cell harvests of biotherapeutic products derived from CHO cell culture has been reported (35–37), there is no evidence that biological products released for distribution have been contaminated with rodent viruses. If present, such viruses were detected during quality-control testing in compliance with GMP prior to release. In addition, it is important to note that there have been no reported cases of transmission of an infectious agent to recipients of recombinant protein products manufactured in animal cells.

Insect cells have recently been used for vaccine production, and various insect cell lines may be used for the production of biologicals in the future. Insect viruses tend to be ubiquitous in many insect cell lines and are generally unknown and/or uncharacterized. Many insect cell lines have endogenous transposons and retrovirus-like particles, and some are positive in product-enhanced reverse transcriptase (PERT) assays.

HDCs have been used for vaccine production for many years. Although concern was initially expressed about the possibility of such cells containing a latent pathogenic human virus, no evidence for such an endogenous agent has been reported, and vaccines produced from this class of cell substrate have proven to be free from viral contaminants.

In light of the differing potential of the various types of cells mentioned above for transmitting viruses that are pathogenic in humans, it is essential that the cells being used to produce biological products should be evaluated as thoroughly as possible with respect to infectious agents.

When DCLs, SCLs or CCLs are used for production, a cell bank system should be used and the cell banks should be characterized as specified in this document. Efforts to identify viruses by testing for viral sequences or other viral markers, especially those not detectable by other means, constitute an important part of the evaluation of cell banks in addition to the standard tests that have been in place for many years.

When cell lines of rodent or avian origin are examined for the presence of viruses, the major emphasis in risk assessment should be on the results of studies in which transmission to target cells or animals is attempted. Risk to human recipients should not be assessed solely on ultrastructural or biochemical/biophysical evidence of the presence of viral or viral-like agents in the cells.

The overall manufacturing process – including the selection and testing of cells and source materials, any purification procedures used, and tests on intermediate or final products – should ensure the absence of detectable infectious agents in the final product. When appropriate, validation of purification procedures should demonstrate adequate reduction of relevant model viruses, with a significant safety factor (14). This is usually required for recombinant protein products.
There may be as yet undiscovered microbial agents for which there is no current evidence or means of detection. As such agents are identified, it will be important to consider whether to re-examine cell banks for their presence. In general, it is not a practice consistent with GMP to retest materials that have already been released, so justification would be necessary before such retesting is undertaken. Positive findings should be discussed with the NRA/NCL. Whenever new data are developed with the potential for impact on the quality, safety or efficacy of a biological product, it is the responsibility of the manufacturer to provide NRAs with all the relevant data and information that are currently available. This should include confirmation and evaluation of the finding, the manufacturer’s own risk assessment and an investigational and action plan, in order to facilitate any regulatory action that may be necessary. In addition, new testing methods are likely to be developed and, as they become available and validated, they should be considered by manufacturers and NRAs/NCLs for their applicability to the characterization and control of new animal cell substrates.

5.2.2 Cellular DNA

The issue of rcDNA in biological products has been considered by many groups since the 1980s, and there has been an evolution of consensus on recommendations. The most recent WHO Recommendation (WHO Technical Report Series, No. 878) sets the upper limit of rcDNA at 10 ng per parenteral dose. As stated below, while this value has proved helpful in the past, it does not take into consideration important factors such as the size of the DNA fragments and any potentially inactivating steps in the manufacturing process. Thus, it is important to take into consideration not only the limit of 10 ng per parenteral dose but other factors as well, when determining the acceptable level of rcDNA.

PCCs and DCLs have been used successfully for many years for the production of viral vaccines, and the rcDNA deriving from these cells has not been (and is not) considered to pose any significant risk. However, with the use of CCLs, which have an apparently indefinite lifespan, presumably due to the dysregulation of genes that control growth, and with the ongoing development of products from cells that are tumorigenic or were derived from tumours, the DNA from such cells has been considered to have the theoretical potential to confer the capacity for unregulated cell growth, and perhaps oncogenic activity, upon some cells of a recipient of the biological product. Although the risk of such DNA has been estimated on the basis of certain assumptions and some experimental data, assessing the actual risk of such DNA has not been possible until recently, when preliminary data generated from new experimental systems began to quantify the risk.

The potential risk of DNA arises from both of its biological activities, namely infectivity and oncogenicity. Infectivity could be due to the presence
of an infectious viral genome in the cellular DNA of the cell substrate (39–41). The viral genome could be that of a DNA virus, whether integrated or extrachromosomal, or of a proviral genome of a retrovirus. Both types of viral DNA have been shown to be infectious in vitro and, in several cases, in vivo (39, 40). The oncogenic activity of DNA could arise through its capacity to induce a normal cell to become transformed and perhaps to become tumorigenic. The major mechanism through which this could occur would be the introduction of an active dominant oncogene (e.g. myc, activated ras), since such dominant oncogenes could directly transform a normal cell. Other mechanisms would require that the rcDNA transforms through insertional mutagenesis, and have been considered less likely since the frequency of integration of DNA is generally low (42). The frequency of integration at an appropriate site, such as inactivating a tumour suppressor gene or activating a proto-oncogene, would be correspondingly lower (32).

The 1986 WHO Study Group addressed the risk posed by the oncogenic activity of rcDNA in biological products for human use (12). Risk assessment based on a viral oncogene in an animal model suggested that in vivo exposure to 1 ng of rcDNA, where 100 copies of an activated oncogene were present in the genome, would give rise to a transformational event once in $10^9$ recipients (27). On the basis of this and other evidence available at that time, the Study Group concluded in 1986 that the risk associated with rcDNA in a product is negligible when the amount of such DNA is 100 pg or less per parenteral dose. On the basis of a review of more recent data, those requirements were revised in 1998 to raise the acceptable level of rcDNA to 10 ng per dose.

Studies in mice using cloned cellular oncogenes also suggest that the risk of neoplastic transformation by cellular DNA is probably very low (34, 43). However, more recent data have shown that cloned cellular oncogene DNA can induce tumours in selected strains of mice at levels below 1 ng. In addition, single oncogenes can also be biologically active (44) and can initiate the tumour induction process. Because of these data and the recent evidence that genes encoding for certain micro-RNA species can be oncogenic in vitro (45–48), thus increasing the number of potential dominant cellular oncogenes, the oncogenic risk of DNA needs to be taken into account when tumorigenic cells are considered for use in the production of biologicals. This would be especially important for live attenuated viral vaccines where chemical inactivation of the DNA is not possible and where the only way to reduce the biological activity of DNA would be by nuclease digestion and the reduction in the quantity of DNA.

In addition to its oncogenic activity, the infectivity of DNA should be considered. Since a viral genome, once introduced, could amplify and produce many infectious particles, the infectivity risk is likely to be greater than the
oncogenic risk. The polyoma virus genome is infectious in mice at about 50 pg (49), and a recent report demonstrated that 1 pg of a proviral copy of a retrovirus is infectious in vitro (50). Because such low levels of DNA may be biologically active, the amounts of rcDNA should be factored into safety evaluations when tumorigenic cell substrates are used, especially for live viral vaccines.

Consequently, considerations that need to be taken into account with respect to rcDNA are: (i) any reduction in the amount of the contaminating DNA during the manufacturing process; (ii) any size reduction of the contaminating DNA during the manufacturing process; and (iii) any chemical inactivation of the biological activity of contaminating DNA during the manufacturing process. A product might be considered by an NRA/NCL to have an acceptable level of risk associated with the DNA of the cell substrate on the basis of (i) and/or (ii) and/or (iii) when data demonstrate that appropriate levels have been achieved. For example, data have shown that nuclease digestion of DNA or chemical inactivation of DNA with beta-propiolactone, a virus-inactivating agent, can destroy the biological activity of DNA (38, 50, 51). Therefore, the use of these procedures may provide an additional level of confidence with respect to reduction of DNA risk.

For products such as monoclonal antibodies and subunit vaccines manufactured in tumorigenic cell substrates, it is necessary to demonstrate the clearance (removal and/or inactivation) of DNA by the manufacturing process. This may require validation of the main inactivating or removal steps. For example, data should be obtained on the effects of DNA-inactivating agents under specific manufacturing conditions, so that firm conclusions can be drawn on their DNA-inactivating potential for a given product.

There may be instances where CCL DNA is considered to pose a higher level of risk because it contains specific elements such as infectious retroviral proviral sequences. Under these circumstances, the steps taken to reduce the risks of rcDNA, such as reducing the size of DNA fragments, should be agreed in consultation with the NRA/NCL.

The 1986 WHO Study Group stated that the risks for rcDNA should be considered to be negligible for preparations given orally. This conclusion was based on the finding that polyoma virus DNA was not infectious when administered orally (49). For such products, the principal requirement is the elimination of potentially contaminating viruses. Recently, additional data have been published on the uptake of DNA via the oral route (52). These studies demonstrated that the efficiency of uptake of DNA introduced orally was significantly lower than for that introduced intramuscularly. Nevertheless, the specifics of the manufacturing process and the formulation of a given product should be considered by the NRA/NCL.
With respect to the efficiency of DNA uptake via the nasal route, no data have been published comparing this route with parenteral routes. However, data suggest that uptake via the intranasal route is less efficient than by the intramuscular route (53). Limits for a specific product should be set in consultation with the NRA/NCL.

In general, acceptable limits of rcDNA for specific products should be agreed in consultation with the NRA/NCL, taking into consideration the characteristics of the cell substrate, the intended use of the biological product and, most importantly, the effect of the manufacturing process on the size, quantity and biological activity of rcDNA fragments. In general, it has been possible to reduce rcDNA in biotechnological products to <10 ng per dose, and in many cases to <10 pg per dose, because they can be highly purified. Quantitative PCR for short amplicons has been used to determine total residual DNA levels as well as residual DNA fragment size distribution. It should be noted that other methods may give different results for small fragments or for DNA that has been treated with inactivating agents. Whatever methods are used should be validated. Some products, especially certain live viral vaccines, are difficult to purify without a significant loss in potency, so the amount of rcDNA in those final products may be significantly higher than 10 ng per dose. Such cases are considered to be exceptional and should be discussed with the NRA/NCL.

5.2.3 Cellular RNA

While protein-coding RNA has not been considered to be a risk factor for biological products, owing to the unstable nature of RNA and the lack of mechanisms for self-replication, the recent description of small non-coding RNA molecules – microRNA (miRNA) – that are more stable and have the capacity to modulate gene expression may necessitate a reassessment. Whether these miRNA molecules can be taken up by cells in vivo is unknown. However, as previously stated, because certain miRNA genes can be oncogenic, DNA containing such sequences may need to be considered along with oncogenes, when assessing the risk of rcDNA (see section B.9 on Oncogenicity). Because this is an evolving area of research, no conclusions can be made regarding the risk of miRNA and no recommendations are made to control miRNA at this time.

5.2.4 Growth-promoting proteins

Growth factors may be secreted by cells that are used to produce biologicals, but the risks from these substances are limited since their growth-promoting effects are usually transient and reversible, they do not replicate, and many of them are rapidly inactivated in vivo. In exceptional circumstances, growth factors may contribute to oncogenesis, but even in these cases, the tumours apparently remain
dependent upon continued administration of the growth factor. Therefore, the presence of known growth factor contaminants at ordinary concentrations does not constitute a significant risk in the preparation of biological products manufactured in animal cell cultures. However, some SCLs may secrete higher levels and more potent factors than CCLs. This should be taken into account when designing characterization studies. The manufacturing process should be designed to address any safety issues that are identified.

**Part A. General recommendations applicable to all types of cell culture production**

**A.1 Good manufacturing practices**

The general principles of GMP for biologicals should be in place. Requirements or recommendations have been made by NRAs (e.g. the European Medicines Agency (EMA), the United States Food and Drug Administration (FDA)) and other groups (e.g. the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH)). GMP should be applied from the stage of cell banking onwards.

In the preparation of a cell substrate, it is considered best practice to establish tiered master and working cell banks (see section A.5.2), to ensure a reliable and consistent supply of cells that can be fully characterized and safety tested prior to use for production. By definition, PCCs cannot be subjected to such banking regimens. However, some manufacturers have utilized pooled and cryopreserved primary cultures, which enable completion of lot release testing as in a tiered banking system. The strategy for delivery of primary cells or primary cells recovered from cryopreservation should be based on the quality and safety that can be assured for the final product according to the overall manufacturing and control processes involved.

**A.2 Principles of good cell culture practice**

**A.2.1 Understanding the cells and the culture system**

In all aspects of sourcing, banking and preparing cell cultures, the principles of good cell culture practice should be observed (e.g. (54, 55) for SCLs). Fundamental features to be considered in the development of cell cultures for production or testing are:

- authenticity, including identity, provenance and genotypic/phenotypic characteristics;
- absence of contamination with another cell line;
- absence of microbiological contamination;
- stability and functional integrity on extended in vitro passage.

An important basic principle for all types of cells is that the donor should be free of transmissible diseases or diseases of uncertain etiology, such as CJD for humans and BSE for cattle. The NRA/NCL may allow specific exceptions concerning donor health (e.g. myeloma, other tumour cells).

Cells in culture may change their characteristics in response to changes in culture conditions or on extended passage under the same culture conditions. The four cell culture types (PCC, DCL, SCL, CCL) used in manufacture differ in their potential stability. Thus, characterization approaches may need to be adapted to reflect these differences.

Cell cultures grow in an in vitro environment that is substantially different from the conditions experienced by cells in vivo, and it is not unexpected that they may be susceptible to change or alteration as a result of in vitro culture and processing. It is important to be conscious of the variation that may arise in the cell culture environment, as cells may undergo subtle alterations in their cell biology in response to such changes. It is therefore necessary to try to control key known variables that could have significant impact on cell culture. Medium and specific additives (serum, growth factors, amino acids and other growth-promoting compounds) should, where possible, be specified in terms of chemical composition and purity. Where relevant, the biological activity of the medium and the additives should be determined before use. New batches of reagents for cell culture should be supplied with certificates of analysis and origin, to enable their suitability to be evaluated against the established specification. The use of serum or other poorly defined reagents is not recommended in the production of new biologicals from cell culture, and chemically defined alternatives should be sought wherever possible. However, given that our current understanding of cell biology is not complete, the benefits that defined media bring in the form of higher reproducibility and reduced risk must be balanced against the potential effects of inadequacies of defined culture systems that may not meet the full biological needs of cells. Where complex biological reagents such as fetal bovine serum (FBS) remain necessary, they should be carefully controlled whenever possible by pre-use selection of batches. Such careful selection also should apply, where relevant, to cell culture surfaces using specified culture vessels or surface coatings.

Variation in physical culture parameters – such as pH, temperature, humidity and gas composition – can significantly influence the performance and viability of cells and should be specified with established tolerances, and the relevant equipment calibrated and monitored. In addition, any culture reagents prepared in the laboratory should be documented, controlled for quality and released against an established specification.
A.2.2 **Manipulation of cell cultures**

In vitro processing of cells can introduce additional physical and biochemical stresses that could have an influence on the quality of the final product. Care should be taken to minimize manipulations, taking into consideration the specifics of the manufacturing process. In all cases, a consistent process should be demonstrated.

A.2.2.1 **Detachment and subculture**

Detachment solutions may adversely affect the cells if exposure is not minimized. Cell harvesting and passaging procedures should be carried out in a reproducible way that ensures consistency in the confluency of cells when harvested, in incubation times, temperature, centrifugation speeds and times, and in post-passage viable cell seeding densities.

A.2.2.2 **Cryopreservation**

(see section A.5.1)

A.2.2.3 **Introduction of contamination**

The microbiological status of the donor individual, colony, herd or flock is an important consideration in the establishment of PCCs. In order to avoid catastrophic failure of the production process and to avoid infectious hazards for the recipients of products, it is important to minimize the opportunities for contamination of cell cultures. Therefore, cell manipulation and open processing steps should be minimized, taking into consideration the specifics of the manufacturing process. It is critical to adopt a rigorous aseptic technique and to provide appropriate environmental controls and air quality for cell culture processing and the preparation of growth media. The presence of any antimicrobial in a biological process or product is discouraged, although a notable exception is that antibiotic(s) and antifungal(s) may be required for primary cell cultures. Additionally, antibiotics may be used in some cell line selection systems. Where antibiotics have been used, sterility-testing procedures should take into account the potential inhibitory effects of the antibiotic on contaminating organisms. Penicillin or other beta-lactam antibiotics should not be present in production cell cultures.

A.2.3 **Training and staff**

Training in all cell culture processes is vital to ensure that correct procedures are adhered to under GMP. Staff should be trained in the underlying principles of cell culture procedures, to give them an understanding of cell culture processes that will enable them to identify events and changes that could influence the quality of
cells (54). Key procedures on which training in good cell culture practice should focus include the passaging of cells, preparation of sterile media, and maintenance and use of biological safety cabinets, incubators and cryopreservation.

Cell cultures should be prepared by staff who have not, on the same working day, handled animals or infectious microorganisms. Furthermore, cell cultures should not be prepared by staff who are known to be suffering from a transmissible infection. The personnel concerned should undergo a “return to work” assessment to evaluate any residual risk.

A.2.4 Cell line development and cloning

Wherever a cell culture has passed through a process that may have a significant influence on its characteristics, such as tumorigenicity, it should be treated as a new (i.e. different) cell line and should be renamed with a suffix or code to identify this. An MCB should then be prepared from the “post-treatment” culture. Treatments that may require such rebanking include cell cloning and genetic manipulation. Any change(s) to the cell culture process should be demonstrated not to affect product quality and should be discussed with the NRA. In the manufacture of monoclonal antibodies, cloning of hybridoma cultures is particularly important in order to ensure that a single product is generated, since inclusion of more than one hybridoma cell type could lead to a mixture of different antibody specificities and classes being present.

The details of cloning and selection may vary according to the practices of individual manufacturers and should be discussed with the NRA/NCL.

In the early stages of cell line development, a number of different recombinant vector systems and cell lines may be used. This will essentially be a research activity, but the cell lines and vectors should originate from well-characterized and qualified sources and the cells from an appropriately qualified seed stock or MCB, which will usually be “in-house” host cells and vectors. The most promising cell–vector combination will then be used to generate a large number of clones (from a few hundred to thousands) after transfecting the culture with rDNA. Typically, these clones will be screened on the basis of their productivity, and a number with the highest productivity (10–50) will be taken forward for further evaluation. Further testing will then be used to select a small number (1–5) for establishment as small pre-master cell banks, and a final selection will be made – often based on stability characteristics and amenability to scale-up – before finally an MCB and WCB are generated. Throughout the process, only well-characterized and traceable growth media and other critical reagents will be used (usually the same as for the MCB), and cryopreserved stocks of all working clones will be made at appropriate stages in the development process (see Figure A3.1).
Annex 3

Figure A3.1
Simplified outline of the development of a genetically modified cell line

In the process of cloning a cell culture, single cells should be selected for expansion. The cloning procedure should be carefully documented, including the provenance of the original culture, the cloning protocol and reagents used. Cloning by one round of limiting dilution will not necessarily guarantee derivation from single cells; additional subcloning steps should be performed. Alternatively, or in addition to limiting dilution steps, the cloning procedure can include more recent technology such as single-cell sorting and arraying or colony-picking from dilute seeds into semi-solid media. In any case, the cloning procedure should be fully documented, with details of imaging techniques and/or appropriate statistics. For proteins derived from transfection with recombinant plasmid DNA technology, a single fully documented round of cloning is sufficient, provided that product homogeneity and consistent characteristics are demonstrated throughout the production process and within a defined cell age beyond the production process.

It is important to document accurately the establishment of each clone, which should also have a unique reference. Cryopreserved seed stocks of a significant number of clones should be established at an early stage. The clones can then be compared in parallel with the parental culture, to establish candidate clones with the best overall characteristics for delivery of the desired product. The criteria used in the evaluation of the clone selected for production should include genomic and phenotypic stability, growth rate, achievable product levels, and integrity/stability of the product. The evaluation of early candidate clones should generate sufficient information for the manufacturer to make an informed decision on the selection of the most promising clone(s) for further development.
Where genetically engineered cell clones are under evaluation, these criteria should also include the stability of integrated rDNA. The details of this process could vary due to a number of factors, including the nature of the host cell, the desired characteristics of the product, and the manufacturer’s local procedures.

It is important to bear in mind that, even following single-cell cloning, epigenetic variation may result in a cloned culture showing evidence of heterogeneity (i.e. more than one clone). This should not preclude the use of such a culture for production unless there are indications of instability that could affect the quality and/or safety of the final product.

A.2.5 Special considerations for neural cell types

Agents causing TSEs have been propagated in certain cells. At the time of writing, the phenomenon has been observed only with very specific pairs of agents and cells; no cell line has been identified that will replicate all agents, although one has been described that seems to be infectable by many strains of scrapie. Although the phenomenon is unpredictable, if the line does not express the prion protein (PrP), it may be assumed to be impossible to infect it, and experience to date suggests that infection is not commonly observed or easy to maintain. On the other hand, the cell types that can be infected include fibroblastic lines as well as neuronal cells. The cells have usually been of murine origin, because the infecting agents are usually mouse-adapted scrapie. The fact that certain cells can be infected with certain agents is proof of the principle that cell lines may be infected; thus, exposure of cells to sources potentially contaminated with the agents is a concern. Since the scale of the risk is difficult to judge, it is recommended that, with respect to safety considerations and TSEs, attention should focus on the selection and documentation of the cell culture reagents and other materials that come into intimate contact with the cells, in order to provide assurance that they are not contaminated. Strategies to accomplish this are given in section B.11.4.

A.3 Selection of source materials

A.3.1 Introduction

All materials should be subjected to risk assessment and testing when necessary – particularly raw materials derived from humans and animals, which can be a primary source for the introduction of adventitious agents into the production of biologicals. Careful attention should be paid to sourcing, production, handling, testing and quality control. All cell culture materials of biological origin that come into intimate contact with the cells – during the establishment of cell cultures, derivation of a new cell line (if any), banking procedures (if any) and production – should be subject to appropriate tests, as indicated by risk assessment, to establish
quality and freedom from contamination by microbial agents and to evaluate acceptability for use in production. It is important to evaluate the microbiological risks represented by each human- and animal-derived reagent used in a cell culture production process. The evaluation should address: (i) geographical origin; (ii) the species of origin; (iii) general microbiological potential hazards, including a consideration of the medical history for human-derived reagents; (iv) the husbandry/screening of donor animals; (v) testing performed on the product, including certificates of analysis (if any); and (vi) the capacity for the preparation, purification and sterilization procedures (if any) used to remove or inactivate contaminants (56). Other reagents of biological but non-animal origin may also present risks to product safety, and these are discussed further in section A.3.4.

Recombinant protein technology now provides many materials that were formerly derived directly from animal or human sources. While this eliminates obvious virological risks from donors, the manufacturing process used for the recombinant proteins should be analysed to identify any materials of biological origin and any associated hazards that may need to be addressed.

The NRA/NCL should approve the source(s) of animal-derived raw materials such as serum and trypsin. These materials should comply with the guidelines on tissue infectivity distribution of TSEs (57). The materials should be subjected to appropriate tests for quality and freedom from contamination by microbial agents, to evaluate their acceptability for use in production. Their origin should be documented to ensure that the sources are from geographical regions with acceptable levels of microbiological risk (e.g. freedom from foot-and-mouth disease virus or BSE). In addition, documentation should be gathered on their manufacturing history, production, quality control and any final or supplementary processing that could affect quality and safety (such as blending and aliquoting of serum batches). Controls should be in place to prevent cross-contamination of one material with another (e.g. bovine material in a porcine product).

The reduction and elimination from the manufacturing process of raw materials derived from animals and humans is encouraged, where feasible.

For some human- and animal-derived raw materials used in the cell culture medium, such as insulin or transferrin, validation of the production process for the elimination of viruses can substitute for virus detection tests, when justified.

Animal-derived reagents such as trypsin and serum, which would be substantially damaged or destroyed in physical sterilization processes, including heat and irradiation, present the most likely microbiological hazards to cell culture processes. Batches of reagents, such as trypsin and bovine serum, have been known to contain *Mycoplasma* species and sometimes more than one viral contaminant. Certain contaminants have also been shown to infect cells
in culture. The processing environment is a common source of microbiological contamination and should be controlled to minimize this risk and to prevent growth of contaminants.

A.3.2 Serum and other bovine-derived materials used in cell culture media

The source(s) of serum of bovine origin should be approved by the NRA/NCL. The responsibility for ensuring the quality of the serum used in the manufacture of cell banks and biologicals rests with the manufacturer of the biologicals. Quality can be ensured in more than one manner. The manufacturer may conduct testing for adventitious agents and perform inactivation of the serum after purchase from the serum manufacturer. Alternatively, the manufacturer may qualify the serum vendor and purchase serum from suppliers only after conducting thorough and ongoing audits of the serum suppliers to ensure that they have properly performed the manufacture, quality control and validation necessary to achieve the level of serum quality required for the biological being produced. In some cases, certificates of analysis may then be considered sufficient. Some combination of these approaches might be optimal, and the strategy taken should be considered when evaluating risk. Consultation with the NRA/NCL may also be advisable.

Serum and other bovine-derived materials should be tested for adventitious agents such as bacteria, fungi, mycoplasmas and viruses, prior to use in the production of MCBs and WCBs and in the manufacture of biologicals. Particular consideration should be given to those viruses that could be introduced from bovine-derived materials and that could be zoonotic or oncogenic (e.g. bovine viral diarrhoea virus (BVDV), bovine polyoma virus, bovine circoviruses, rabies virus, bovine adenoviruses, bovine parvovirus (BPV), bovine respiratory syncytial virus (BRSV), infectious bovine rhinotracheitis virus (IBR), bovine parainfluenza virus type 3 (BPIV3), reovirus 3 (REO3), Cache Valley virus, bluetongue virus (BTV) and epizootic haemorrhagic disease virus). Consideration should also be given to risk-mitigation strategies, such as inactivation by heat or irradiation, to ensure that adventitious agents that are not detected in the manufacture and quality control of the serum will be inactivated to a degree that is acceptable to the NRA/NCL. If irradiation or other inactivation methods (e.g. heat sterilization) are used in the manufacture of the serum, the tests for adventitious agents should be performed prior to inactivation, to enhance the opportunity for detecting contamination. If evidence of viral contamination is found in any of the tests, the serum is acceptable only if the virus is identified and shown to be present in an amount that has been shown in a validation study to be effectively inactivated. If evidence of viral contamination is found in any tests on serum that is not to be subjected to a virus inactivation or removal procedure, the serum would not generally be acceptable. If the manufacturer chooses to use serum that has not been inactivated, thorough testing of the serum for
adventitious agents, using current best practices, should be undertaken. If any agents are identified, the cell banks made in this manner must be shown to be free of the identified virus(es).

If irradiation is used, it is important to ensure that a reproducible dose is delivered to all batches and to the component units of each batch. The irradiation dose must be low enough for the biological properties of the reagents to be retained, while being high enough to reduce virological risk. Thus, irradiation delivered at such a dose may not be a sterilizing dose.

If serum was used in the establishment or passage history of the animal cell substrate prior to banking by the manufacturer, the cell bank (MCB or WCB) and/or cells at or beyond the level of production should be tested for adventitious agents of the species (e.g. bovine) of serum used in the establishment and passage history of the cell substrate. If serum is not used in the production of the subsequent stages, then this testing would not need to be repeated on those subsequent stages, once the cell bank has been tested and considered free of bovine (or whichever species of serum was used) adventitious agents.

Methods used to test for bovine viruses should be approved by the NRA/NCL. Details of the methods are provided in Appendix 1. Infectivity assays are used as the primary screening method and have resulted in the detection of BVDV, REO3, Cache Valley virus, BTV and epizootic haemorrhagic disease virus, among others. However, it should be noted that, in general, the infectivity screening assay methods described here do not readily detect some of the viruses (e.g. bovine polyomaviruses) that can be frequent contaminants of serum. Additional methods may need to be considered, such as the nucleic acid amplification technique (NAT), although the presence of viral genomic sequences is not necessarily indicative of infectious virus. In those cases, specific infectivity assays designed to detect the virus of concern (e.g. bovine polyomavirus) may need to be considered.

A second factor in screening serum is the limited sample volume used, compared with the batch size (which may be of the order of 1000:1) that comes from the pooling of serum from many animals. Consequently, infectious viruses may be missed in the serum lot testing, and consideration should be given to direct screening of the cell bank for bovine viruses. These assays could include, in addition to the general screening procedure, NAT for the presence of bovine viruses that may infect the cell substrate but undergo abortive and/or transforming infections. Virus families of particular concern in this regard include polyomaviruses, herpesviruses, circoviruses, anelloviruses and adenoviruses.

General screening assays for the detection of infectious viruses in serum or cell substrates involve the use of at least one indicator cell line, such as bovine turbinate cells, that is permissive for the replication of BVDV. A second cell line,
such as Vero, should also be employed to broaden the detection range. Before initiating screening, it may be necessary to evaluate the serum for the presence of antibody, particularly to BVDV, that could mask the presence of infectious virus.

Indicator cells are cultured typically in the presence of the serum under test for 21–28 days, passaging the cells as necessary. During this period, the cells are regularly examined for the presence of CPE indicative of virus infection. At the end of the observation period, which should not be less than 7 days after the last subculture, the cells are stained to detect CPE that may have been missed during observation of the living cells. Additional end-point assays should include haemadsorption and haemagglutination at both 4 °C and a higher temperature such as 20–25 °C and also immunofluorescence assays (IFAs) for specific viruses. Appropriate controls should be used for each assay – such as BPIV3 for haemadsorption. IFAs are particularly important for BVDV, since non-cytopathic BVDV may be present in the serum. IFA end-points are also used to detect other viruses that may be determined by geographical considerations – such as adenoviruses, BPV, BTV, BRSV, REO3 and unlikely but serious contaminants like rabies virus.

If serum from another species is used (i.e. other than bovine), the NRA/NCL should be consulted about acceptable testing methods for that species.

A.3.3 Trypsin and other porcine-derived materials used for preparing cell cultures

Trypsin used for preparing cell cultures should be tested for cultivable bacteria, fungi, mycoplasmas and infectious viruses, including bovine or porcine parvoviruses, as appropriate. The methods used to do this should be approved by the NRA/NCL.

In some countries, irradiation is used to inactivate potential contaminant viruses. If irradiation is used, it is important to ensure that a reproducible dose is delivered to all batches and the component units of each batch. The irradiation dose must be low enough for the biological properties of the reagents to be retained, while being high enough to reduce virological risk. Thus, irradiation cannot be considered a sterilizing process.

The quality of the trypsin, like serum, is the responsibility of the biologicals manufacturer (see section A.4.2). Recombinant trypsin is available and should be considered. However, it should not be assumed that recombinant trypsin is free from the risk of contamination and it should be subject to the usual considerations for any reagent of biological origin (see section A.4.1).

Like serum batches, which are derived from many animals, trypsin batches are also prepared from the pancreases of many animals. Most batches of porcine trypsin contain genetic sequences of porcine parvovirus 1 and porcine circoviruses and should therefore be treated in a manner accepted by the
NRA/NCL in order to inactivate any virus that may potentially be present. It is acknowledged, however, that these viruses are relatively resistant to inactivation (58). If trypsin from another species is used, the NRA/NCL should be consulted regarding acceptable testing methods.

General screening assays for the detection of infectious porcine viruses in trypsin or cell substrates involve the use of at least one indicator cell line, such as porcine testes cells or Vero cells, that is permissive for the replication of porcine viruses. Typically, indicator cell cultures would be incubated for 14 days with a subculture on to fresh test cells for an additional 14 days. Specific end-point detection methods such as IFA or PCR may be required, in addition to periodic observation for CPE throughout the culture period and more general end-point detection methods such as haemadsorption and/or haemagglutination.

If trypsin has been used in the establishment or passage history of the animal cell substrate prior to banking by the manufacturer, the cell bank (MCB or WCB) should be tested for porcine parvovirus or for appropriate adventitious viruses relevant to the species of origin of the trypsin used. If trypsin is not used in the production of the subsequent stages, then this testing would not need to be repeated on those subsequent stages once the cell bank has been tested and is considered free of porcine parvovirus (or relevant agents). Consideration should be given to screening for other agents such as porcine circoviruses. Molecular methods such as PCR may be used for such purposes.

Testing of cells exposed to trypsin or of other porcine-derived materials might entail testing for more than porcine parvovirus or porcine circoviruses. For instance, it may be appropriate to test for porcine adenovirus, transmissible gastroenteritis virus, porcine haemagglutinating encephalitis virus, BVDV, reoviruses, rabies virus, porcine anellovirus, porcine hokovirus, porcine bocavirus, porcine hepatitis E virus, porcine reproductive and respiratory syndrome virus, encephalomyocarditis virus and potentially other viruses. Particular consideration should be given to those viruses that could be introduced from the porcine-derived material and that could be zoonotic or oncogenic. Additionally, tests for bacterial and fungal sterility and mycoplasmas should be conducted depending on the type of porcine-derived material. The NRA/NCL should be consulted on this issue.

A.3.4 Medium supplements and general cell culture reagents derived from other sources used for preparing cell cultures

The quality of medium supplements derived from other species should be controlled from the perspective of adventitious agents. Consideration should be given to whether recombinant-derived medium supplements were exposed to animal-derived materials during their manufacture and, if so, they should be evaluated for the potential to introduce adventitious agents into the manufacture of the cell banks and biological products. Testing for adventitious agents should
assess viruses relevant to the species from which the supplement was derived. The NRA/NCL should be consulted on this issue.

Medium supplements should generally not be obtained from human source materials. In particular, human serum should not be used. However, in special circumstances, and in agreement with the NRA/NCL, the use of human-derived supplements may be permitted. If human serum albumin is used at any stage of product manufacture, the NRA/NCL should be consulted regarding the requirements, as these may differ from country to country. As a minimum, human serum albumin should meet the revised Requirements for biological substances no. 27 (59), as well as the Guidelines on tissue infectivity distribution of TSEs (57).

Recombinant human albumin is commercially available and should be considered. However, it should not be assumed to be free of risk of contamination and should be subject to the usual safety considerations for any reagent of biological origin (see section A.3.1).

As for other cell culture reagents, it is important to establish traceability and to assess and reduce microbiological risks as described in section A.3.1.

A variety of cell culture reagents of biological origin is available. These reagents are derived from non-animal sources, including a range of aquatic organisms, plants and algae. In such cases, the exact hazards involved may be uncertain and unfamiliar. The microbiological risks may be substantially different from those involved in animal-derived reagents (see sections A.3.1–A.3.3), and other hazards may arise – such as immunogenic, mitogenic and allergenic properties of the reagent and its components. Plant-derived material may, for instance, carry an increased risk of mycoplasma and mycobacteria contamination.

A.4 Certification of cell banks by the manufacturer

It is vital that the manufacturer secures a body of information on the cell substrate, demonstrating clearly the origin or provenance of the culture and how the cell banks intended for production (MCB and WCB) were established, characterized and tested. This should provide all the information required to demonstrate the suitability of that cell substrate and the established cell banks for the manufacture of biological products.

A.4.1 Cell line data

Each new cell line should have an associated body of data, which will increase as the cell line is established and developed for manufacturing purposes. This dataset is vital for demonstrating the suitability for use of the cells and should provide information on cell provenance (donor information and any relevant details on ethical procurement), cell line derivation, culture history, culture conditions (including reagents), early-stage safety evaluation data, banking and
cell bank characterization, and safety testing. This information should be made available to the NRA/NCL for approval of the cells used in manufacture.

A.4.2 **Certification by the manufacturer of primary cell cultures**

Full traceability of PCCs should be established to the animals of origin, husbandry conditions, veterinary inspection, vaccinations (if any), procedures for administering anaesthesia and cell harvesting, reagents and procedures used in the preparation of primary cultures, and the environmental conditions under which they were prepared. Extensive testing should be performed and should be documented.

It is important to define the batch or lot of cells used in each individual manufacturing process. For production purposes, a batch or lot is a culture of primary cells derived from single or multiple animals that has been subjected to a common process of tissue retrieval, disaggregation and processing, leading to a single-culture preparation of cells. Lots may be prepared by harvesting and pooling cells in different ways, but the cell-processing procedures should be reproducible. It is especially important to monitor cultures carefully for evidence of adverse change in the cell culture and of microbiological contamination. Prior to any culture pooling, cells should be examined for acceptability for production. Acceptability criteria should be established and should include testing for microbiological contamination and the general condition of the cells (e.g. morphology, number, viability of the cells). Failure to detect and eliminate atypical (i.e. potentially virally infected) or grossly contaminated cells will put the entire production run at risk and could compromise the safety of the product. Cells showing an unacceptably high proportion of dead or atypical cells should not be used, and microbiological testing should ideally be completed and passed before the cells are used.

The preparation of cell lots for manufacture should be carefully documented, to provide full traceability from the animal donor(s) to production. Any pooling of cells should be clearly recorded, as should any deviation from standard operating procedures, as required by GMP. In addition, any observations of variation between batches should be recorded, even where such observations would not necessarily lead to rejection of those batches. Such information may prove valuable in ongoing optimization and improvement of the production process.

A.4.3 **Certification by the manufacturer of diploid, continuous and stem cell lines**

All cell lines used for production of biologicals should have data available, as indicated in section A.4.1. The original PDL (or passage number, if the PDL is unknown) of the cell seed should be recorded. For cell lines of human origin, the medical history of the individual from whom the cell line was derived should, if
possible, be evaluated in order to better assess potential risks and the suitability of the cell line.

For SCLs, morphology continues to be an important characteristic. Representative images and immune-phenotypic profiles of undifferentiated and differentiated cells should be available for comparison.

A.5 Cryopreservation and cell banking

A.5.1 Cryopreservation

When cells are banked, the successful preservation of cells at ultra-low temperatures is critical to the efficient delivery of good quality cultures (i.e. high-viability cultures with the required characteristics). The need to prepare large stocks of frozen vials of cells for cell banks is especially challenging, and a number of key principles should be adopted.

- A method that meets current best practice for cell culture preservation should be used (see, for example (60)).
- The cooling profile for the cells being frozen should be defined, and the same cooling process should be used for each separate preservation process (i.e. the standard operating procedure should include documentation of the cooling process in the batch record).
- Each preservation process should be recorded.
- As a general guide, only cell cultures that are predominantly in the exponential phase of growth should be used. Cells in such cultures tend to have a low ratio of cytoplasm to nucleus (v/v) and should be more amenable to successful cryopreservation. It is unwise to use cells predominantly in the “lag” phase very early after passage, or in the “stationary” phase when the culture has reached its highest density of cells.
- For each bank, cells pooled from a single expanded culture (i.e. not from a range of cultures established at different times post-seeding or different PDLs) should be used and mixed prior to aliquoting to ensure homogeneity.
- The number of cells per vial should be adequate to recover a representative culture (e.g. 5–10 × 10⁶ in a 1 ml aliquot).
- For new cell banks, antimicrobials should not be used in cell cultures to be banked, except where this can be justified for early PDL cultures, which may carry contamination from tissue harvesting or recombinant cells which require antibiotic selection, and when necessary for the genetic stability of recombinant cell lines. If antimicrobials are used, they should not be penicillin or any other beta-lactam drug.
■ When a stock of cells has been frozen, a sample should be recovered to confirm that it has retained viability and the results have been recorded. It is also important to establish the degree of homogeneity within the cell bank. Recovery of a sufficient percentage (e.g. 1%, or as recommended by the NRA) of vials representative of the beginning, middle and end of the cryopreservation process should be demonstrated to give confidence in the production process based on the use of that cell bank. Ultimately, stability (see section B.3) and integrity of cryopreserved vials is demonstrated when the vials are thawed from production and shown to produce the intended product at scale (see also section B.7).

■ Cell bank cryostorage vessels should be monitored and maintained to enable demonstration of a highly stable storage environment for cell banks. Access to such vessels may cause temperature cycling, which in extreme cases can cause loss of viability. It is prudent to establish a stability-testing programme that involves periodic recovery of cells where the frequency of recovery relates to the risk of temperature cycling. New developments in remote monitoring of individual vials may, in the future, eliminate the need for stability testing.

A.5.2 Cell banking

When DCLs, SCLs or CCLs are used for the production of a biological, a cell bank system should be in place. Cell banks should be approved by and registered with the NRA/NCL as part of the product approval process. The source of cells used in cell banking and production is a critical factor in biological product development and manufacture. It is highly desirable to obtain cells from sources with a documented history and with traceability to the originator of the cell line.

After a sample of the original seed stock is obtained, an early-stage pre-master bank of just a few vials should be established. One or more vials of the pre-master bank are used to establish the MCB. The WCB is derived by expansion of one or more containers of the MCB. The WCB should be qualified for yielding cell cultures that are acceptable for use in manufacturing a biological product.

When using early PDLs from primary cultures for production processes, the preparation of a cell bank should be considered on a case-by-case basis. This approach has significant benefits: it gives great flexibility in the timing of the production process, permits quality control and safety testing to be completed prior to use, and reduces the overall burden of testing required in the process.

Cell banks should be characterized as specified in Part B of these recommendations, and according to any other currently valid and future guidance published by WHO. The testing performed on a replacement MCB (derived from the same cell clone or from an existing MCB or WCB) is the same as for the initial MCB, unless a justified exception is made. Efforts to detect contaminating viruses
and other microbial agents constitute a key element in the characterization of cell banks.

Having been cryopreserved by qualified methods (see section A.5.1), both the MCB and WCB should be stored frozen under defined conditions, such as in the vapour or liquid phase of liquid nitrogen. The location, identity and inventory of individual cryovials or ampoules of cells should be thoroughly documented. It is recommended that the MCB and WCB each be stored in at least two widely separated areas within the production facility and/or in geographically distinct locations, in order to ensure continued ability to manufacture products in the event of a facility catastrophe. When cryopreserved cells are transferred to a remote site, it is important to use qualified shipping containers and to monitor transfers with probes to detect temperature excursions. All containers are treated identically and, once removed from storage, are not usually returned to the stock. The second storage site should operate under an equivalent standard of quality assurance to that at the primary site.

A.5.3 **WHO reference cell banks (RCBs)**

The principle of establishing RCBs under WHO auspices is one that offers potential solutions to future challenges for the development of vaccines and biotherapeutics in developing regions. However, WHO does not intend that cells supplied to manufacturers from any RCB should be used as an MCB. The purpose of WHO RCBs is to provide well-characterized cell seed material for the generation of an MCB by manufacturers, with the expectation that such MCBs will comply with this guidance document and be fully characterized.

The WHO RCBs provide key advantages for vaccine development worldwide, which include:

- traceability to the origin of cells and derivation of the cell line and materials used in preparation of seed stock;
- being subject to open international scientific scrutiny and collaborative technical investigations into the characteristics of the cells and the presence of adventitious agents;
- the results of characterization are peer-reviewed and published;
- investigations are evaluated under the auspices of WHO expert review and qualified as suitable for use in vaccine production;
- supply of cells free of any constraint related to intellectual property rights on final products;
- a single source of cells with a growing and scientifically and technically updated body of safety-testing data and safe history of use, giving increased confidence to manufacturers, regulators and public policy-makers.
The Vero cell line has been the most widely used continuous cell line for the production of viral vaccines over the past two decades. The WHO Vero RCB 10-87 was established in 1987 and was subjected to a broad range of tests to establish its suitability for vaccine production. This WHO RCB provides a unique resource for the development of future biological medicines where a cell substrate with a history of safe and reliable use is desired. A comprehensive review of the characterization of the WHO Vero 10-87 seed lot was conducted recently, and a detailed overview is provided on the WHO web site (http://www.who.int/biologicals/).

As concluded by an expert review in 2002, the WHO Vero RCB 10-87 is not considered suitable for direct use as MCB material. However, the WHO Vero RCB 10-87 is considered suitable for use as a cell seed for generating an MCB, and its status has changed from “WHO Vero cell bank 10-87” to “WHO Vero reference cell bank 10-87”.

The WHO Vero RCB 10-87 is stored in the European Collection of Animal Cell Cultures (www.hpacultures.org.uk) in the United Kingdom and the American Type Culture Collection (ATCC, www.atcc.org) in the USA. These public service culture collections have distributed ampoules under agreements with WHO to numerous manufacturers and other users. The WHO Vero RCB 10-87 is the property of WHO and there are no constraints relating to intellectual property rights. The WHO Vero RCB 10-87 is available free of charge on application to WHO. However, owing to the limited number of vials remaining, distribution is restricted to use in the production of vaccines and other biologicals. Potential replacement of the WHO Vero RCB 10-87 is currently under consideration.

WHO also has overseen the establishment of seed stocks of MRC-5 for the production of vaccines. The WHO MRC-5 RCB was established in 2007 because of stability issues associated with the original vials of MRC-5 cells, which dated from 1966. This RCB was prepared in a qualified cleanroom environment and was subjected to specified quality-control testing endorsed by the WHO Expert Committee on Biological Standardization.

Part B. Recommendations for the characterization of cell banks of animal cell substrates

B.1 General considerations

Since the 1986 Study Group report, advances in science and technology have led to an expanded range of animal cell types being used for the production of biologicals. In some cases, these new cell types provide significantly higher yields of product at less cost, while in other cases they provide the only means by which a commercially viable product can be manufactured. Many products
manufactured in CCLs of various types have been approved, and some examples are listed in Table A3.1.

### Table A3.1
**Examples of approved biological products derived from CCLs**

<table>
<thead>
<tr>
<th>Product class</th>
<th>Product (disease)</th>
<th>Cell line</th>
</tr>
</thead>
<tbody>
<tr>
<td>Therapeutic</td>
<td>Factor VIII (haemophilia)</td>
<td>CHO</td>
</tr>
<tr>
<td></td>
<td>Factor VIIa (haemophilia)</td>
<td>BHK-21</td>
</tr>
<tr>
<td></td>
<td>Monoclonal antibody (various diseases)</td>
<td>CHO and murine myeloma (NS0 and SP2/0)</td>
</tr>
<tr>
<td>Prophylactic</td>
<td>Poliovirus vaccine</td>
<td>Vero</td>
</tr>
<tr>
<td></td>
<td>Rotavirus vaccine</td>
<td>Vero</td>
</tr>
<tr>
<td></td>
<td>Rabies vaccine</td>
<td>Vero</td>
</tr>
<tr>
<td></td>
<td>Japanese encephalitis vaccine</td>
<td>Vero</td>
</tr>
<tr>
<td></td>
<td>Human papillomavirus vaccine</td>
<td>Sf-9</td>
</tr>
<tr>
<td></td>
<td>Influenza vaccine</td>
<td>MDCK</td>
</tr>
</tbody>
</table>

Many more products are currently in development. Some products use highly tumorigenic cells (e.g. HeLa, and some banks of MDCK) and some involve sources previously unused in production, such as insect cells. Examples are listed in Table A3.2.

### Table A3.2
**Examples of biological products in development derived from CCLs**

<table>
<thead>
<tr>
<th>Product class</th>
<th>Product (disease)</th>
<th>Cell line(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Therapeutic</td>
<td>More than 50% of products in development use CHO or murine myeloma cells as the cell substrate (61). Monoclonal antibodies are generally produced using CHO, SP2/0, PER.C6 and NS0 cells (62).</td>
<td></td>
</tr>
<tr>
<td>Prophylactic</td>
<td>HIV vaccines</td>
<td>CHO, Vero, PER.C6, 293ORF6, HER96, HeLa</td>
</tr>
<tr>
<td></td>
<td>Herpes simplex type 2 vaccine</td>
<td>CHO</td>
</tr>
<tr>
<td></td>
<td>Influenza vaccines</td>
<td>sf9, Vero, PER.C6</td>
</tr>
<tr>
<td></td>
<td>Rabies vaccine</td>
<td>S2</td>
</tr>
</tbody>
</table>
CCLs may have biochemical, biological and genetic characteristics that differ from PCCs or DCLs and that may impose a risk for the recipients of the biologicals derived from them. In particular, CCLs may produce transforming proteins and may contain potentially oncogenic DNA and viral genes. In some cases, CCLs may cause tumours when inoculated into animals. Non-tumorigenic cells (e.g. PCCs and DCLs) had been thought to be intrinsically safer than tumorigenic cells. Where tumorigenic cells have been used in the past (e.g. CHO for recombinant proteins), high degrees of purity have been required, with a special emphasis on reduction in the quantity of DNA. When it was not possible to reduce the amount of DNA to below the detection limit, emphasis has been placed on a reduction in size or other approaches to inactivate rcDNA and rcRNA (e.g. beta-propiolactone for rabies vaccine).

Manufacturers considering the use of CCLs should be aware of the need to develop, evaluate and validate efficient methods for purification, as an essential element of any product development programme. However, a minimally purified product, such as certain viral vaccines (e.g. polio), may be acceptable if produced in a CCL such as Vero, when data are developed to support the safety of the product. Such data would include extensive characterization of the MCB or the WCB and of the product itself.

While tumorigenicity tests have been part of the characterization of CCLs, they comprise only one element in an array of tests, the results of which must be taken into account when assessing the safety of a biological produced in a given cell substrate. For example, if a CCL is positive in a tumorigenicity test, and if the CCL is to be used for the production of a live viral vaccine, an evaluation of the oncogenic potential of the cells may be requested by the NRA/NCL, to characterize the cellular DNA and to detect oncogenic viruses that might be present. Such studies should be discussed with the NRA/NCL.

Evidence should be provided for any animal cell line that is proposed for use as a substrate for the manufacture of a biological product, to demonstrate, to the limits of the assay’s detection capabilities that the CCL is free from cultivable bacteria, mycoplasmas, fungi and infectious viruses, including potentially oncogenic agents. Special attention should be given to viruses that commonly contaminate the animal species from which the cell line is derived, and to cell culture reagents of biological origin. The cell seed should preferably be free of all microbial agents. However, certain CCLs may express endogenous retroviruses. Tests capable of detecting such agents should be carried out on cells grown under cell culture conditions that mimic those used during production, and the levels of viral particles should be quantified. Viral contaminants in an MCB and WCB should be shown to be inactivated and/or removed by the purification procedure used in production. The validation of the purification procedure used is considered essential.
The characterization of any DCL, SCL or CCL to be used for the production of biologicals should include:

- a history of the cell line (i.e. provenance) and a detailed description of the production of the cell banks, including methods and reagents used during culture, PDL, storage conditions, viability after thawing, and growth characteristics;
- the results of tests for infectious agents;
- distinguishing features of the cells, such as biochemical, immunological, genetic or cytogenetic patterns, that allow them to be clearly distinguished from other cell lines;
- the results of tests for tumorigenicity, including data from the scientific literature.

Additional consideration should be given to products derived from cells that contain known viral sequences (e.g. Namalwa, HeLa, 293 and PER.C6).

The recommendations that follow are intended as guidance for NRAs, NCLs and manufacturers, as the minimum amount of data on the cell substrate that should be available when considering a new biological product for approval. The amount of data that may be required at various stages of clinical development of the product should be discussed and agreed with the NRA/NCL at each step of the programme.

B.2 Identity

Cell banks should be authenticated by a cell identification method approved by the NRA/NCL. Wherever practicable, methods for identity testing should be used that give specific identification of the cell line, in order to confirm that no switching or major cross-contamination of cultures has arisen during cell banking and production. A number of the commonly used identity-testing methods are compared in Table A3.3. In the case of human cells, genetic tests such as DNA profiling (e.g. short tandem repeat analysis, and multiple single nucleotide polymorphisms) will give a profile that is at least specific to the individual from whom the cells were isolated. Another test that may be used for human cells is human leukocyte antigen (HLA) typing. Other tests that may be used but tend to be less specific include isoenzyme analysis and karyology, which may be particularly useful where there are characteristic marker chromosomes. However, where more specific genetic markers are available, they should be considered. A small proportion of cell lines – particularly those that are transformed – may show alterations to the expected identity profile. This has been observed
in isoenzyme analysis where, in rare cases, a particular cell line may show a consistently different profile from that expected for the species of origin; it is also a general issue relating to the effect of genetic instability on molecular identity-testing techniques. Such effects in standard technologies are rare but may also arise with the implementation of new techniques. The implications of any unexpected results should be discussed with the NRA/NCL. For recombinant protein products, cell-line identity testing should also include tests for vector integrity, expression plasmid copy number, insertions, deletions, number of integration sites, percentage of host cells retaining the expression system, verification of protein-coding sequences, and protein-production levels.

Table A3.3

<table>
<thead>
<tr>
<th>Technique</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Karyology (especially useful for DCLs and SCLs)</td>
<td>Gives whole chromosomal genome visualization and analysis that can identify species of origin for a very wide range of species using the same methodology.</td>
<td>Results are generally not specific to the individual of origin (i.e. there is usually species specificity), although certain cell lines may have marker chromosomes that are readily recognized.</td>
</tr>
<tr>
<td>Karyology (especially useful for DCLs and SCLs)</td>
<td>Newer methods include spectral karyotyping, which involves the use of probes labelled with fluorescent dyes. The probes paint the chromosomes, yielding different colours in specific areas. Spectral karyotyping is able to detect translocations that are not recognizable by traditional banding methods.</td>
<td>Giemsa banding requires special expertise and is labour intensive. Standard analysis of 10–20 metaphase spreads is insensitive for detecting contaminating cells.</td>
</tr>
<tr>
<td>Isoenzyme analysis</td>
<td>Determination of species of origin within a few hours.</td>
<td>Analysis for 4–6 isoenzyme activities will generally identify the species of origin but is not specific to the individual of origin.</td>
</tr>
</tbody>
</table>
Table A3.3 continued

<table>
<thead>
<tr>
<th>Technique</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
</table>
| DNA profiling using variable number of tandem repeats (VNTR) analysis or other PCR method such as exon-primed intron crossing-PCR (EPIC-PCR), or techniques such as restriction fragment length polymorphism (RFLP) | Short tandem repeats (STR) analysis by PCR is rapid and gives identity specific to the individual of origin.  
Commercial kits are available for a range of human populations.  
The EPIC-PCR method is rapid and gives identity specific to the individual of origin. It provides the advantage of covering a broad spectrum of organisms and cell lines other than human cells. | Some limited, but undefined, cross-reaction of human STR primers for primate cells. |

Applicability

- **Cell banks:** MCB and each WCB
- **Cell types:** DCL, SCL, CCL

B.3 Stability

The stability of cell banks during cryostorage, and the genetic stability of cell lines and recombinant expression systems, are key elements in a successful cell bank programme.

B.3.1 Stability during cryostorage

Data should be generated to support the stability or suitability of the cell substrate and any recombinant expression system or necessary cell phenotype during cultivation to or beyond the limit of production, and to support the stability of the cryopreserved cell banks during storage. The latter may be demonstrated by successful manufacture of WCBs or production lots. Periodic testing for viability is not necessary if continuous monitoring records for storage show no deviations out of specification, and periodic production runs are successful. If banks are used less than once every 5 years, it may be prudent to generate data to confirm their suitability for manufacturing on a schedule that takes into account the storage condition once every 5 years.
Applicability

- **Cell banks:** MCB and WCB
- **Cell types:** DCL, SCL, CCL

B.3.2 Genetic stability

Any form of genetic instability can potentially affect the quality of the final product and it is important to know if the cells in culture are changing in a way that could affect the nature or safety of the product. Any features of the cell lines that might affect quality should be discussed with the NRA/NCL to ensure that tests used by the manufacturer to monitor genetic stability are adequate. The actual tests will vary according to the nature of the product, but the aim is to show consistency in the amount and characteristics of the product derived from cells within a few passages of the MCB or WCB with those derived from an ECB or EOPC. For recombinant protein products, emphasis will be on the protein sequence and post-translational modifications.

For cell lines containing DNA-expression constructs, the stability of these constructs between the MCB/WCB and an ECB or EOPC should be determined. The copy number of the construct and, if relevant, the sites of chromosomal insertion, should also be determined. The latter is accomplished by sequencing into the cellular flanking regions, but methods such as fluorescent in situ hybridization may provide useful additional information, particularly where concatamers of the gene insert are present at individual chromosomal loci. The sequence of the construct within the cells should be determined. With conventional sequencing, a consensus sequence is obtained, but with MPS it is possible to determine the sequence of individual gene inserts or their transcripts.

Where proteins are derived from cells that have not been genetically modified, consistency in the yield and properties of the protein should be evaluated, together with the sequence of the messenger RNA (mRNA) encoding the protein of interest.

Additional characterization of the cell biological processes and responses during cultivation (for instance, using global or targeted gene expression, proteomic or metabolic profiles and other phenotypic markers) might be useful in further developing a broad understanding of the cell substrate.

Appropriate methods should be applied to ensure that cell age is correctly assessed in the event that cell viability falls dramatically at any given step. Losses in viability are reflected in increased cultivation times to reach defined levels of growth.

The stability of cell function in terms of productivity within the production process also may need to be evaluated. Other stability studies may be performed where bioreactor methods are employed, especially where extended culture periods are involved.
Applicability

- **Cell banks**: MCB taken to EOPC/ECB
- **Cell types**: DCL, SCL, CCL

B.4 Sterility

(see section B.11.3.1)

B.5 Viability

A high level of viability of cryopreserved cells is important for efficient and reliable production. Thawed cells should typically have viability levels in excess of 80%, though this is not always achieved and may depend on the cell line. Lower viabilities may still result in suitable growth recovery and in acceptable product qualities. In such cases, the data should be discussed with the NRA/NCL. A range of viability tests is available to measure different attributes of cell function (e.g. membrane integrity, metabolic activity, DNA replication). Under certain circumstances, such as pre-apoptotic cells excluding trypan blue, viability assays may give misleading results and it is important to be aware of the exact information that a particular viability assay provides. Therefore, it is important to evaluate the growth recovery of cryopreserved cells upon thawing.

For certain cell cultures such as hybridomas, where a membrane-integrity test is used, additional cell markers such as indicators of apoptosis should be studied, in order to avoid significant overestimation of viability.

A suitable viability test should be selected for the cell substrate in question and typical test values established for cultures considered to be acceptable (see sections B.6 and B.7). It may also be necessary to select alternative viability assays that are better suited to providing the in-process viability data that are required during production (e.g. lactate dehydrogenase levels in bioreactor systems).

Applicability

- **Cell banks**: MCB and WCB
- **Cell types**: DCL, SCL, CCL

B.6 Growth characteristics

For the development of production processes, the growth characteristics of the production cell line should be well understood, in order to ensure consistency of production. Changes in these characteristics could indicate any one of a range of events. Accordingly, data on growth characteristics – such as viability, morphology, cell-doubling times, cloning and/or plating efficiency – should be
developed. For certain cell substrates, it may be appropriate to apply such tests in homogeneity testing (see section B.7). Experiments to demonstrate homogeneity and growth characteristics may be combined, although the analysis should be carried out separately.

Applicability

- Cell banks: MCB and WCB
- Cell types: DCL, SCL, CCL

B.7 Homogeneity

Each cell culture derived from a container of the WCB should perform in the same way (i.e. within acceptable limits) and should provide the same number of viable cells with the same growth characteristics. In order to ensure this, it is important to recover a proportion of containers from each cell bank and check their characteristics, as indicated in section B.6. The number of containers tested should be discussed with the NRA/NCL and should be broadly in line with the number normally sampled to establish product consistency. Recovery of a sufficient percentage of vials representative of the beginning, middle and end of the aliquoting process should be demonstrated, in order to give confidence in the production process that is based on the use of that cell bank. Ultimately, stability and integrity of cryopreserved vials are demonstrated when the vials are thawed for production and are shown to produce the intended product at scale. Instead of testing a proportion of containers at different stages of the banking process, an alternative strategy to ensure the homogeneity of the banks can be based on the validation of the process method for filling and freezing. Assessment of growth characteristics (see section B.6) and homogeneity testing are commonly combined experimentally; however, the analysis and interpretation of each should be distinct. It may also be appropriate to test the homogeneity of the MCB to assure that future WCBs are consistent with the first WCB.

Applicability

- Cell banks: MCB, WCB
- Cell types: DCL, SCL, CCL

B.8 Tumorigenicity

B.8.1 General considerations

Several in vitro test systems, such as cell growth in soft agar (63) and muscle organ culture (64), have been explored as alternatives to in vivo tests for tumorigenicity.
However, correlations with in vivo tests have been imperfect, or the alternative tests have been technically difficult to perform. Thus, in vivo tests remain the standard for assessing tumorigenicity.

Although WHO Requirements (1) have described acceptable approaches to tumorigenicity testing, a number of important aspects of such testing were not addressed. Therefore, a model protocol has been developed and is appended to this document (see Appendix 2). The major points included in the model protocol are listed below, along with comments on each item.

A new DCL (i.e. other than WI-38, MRC-5 and FRhL-2) should be tested for tumorigenicity as part of the characterization of the cell line, but should not be required on a routine basis.

The tumorigenicity tests that are currently available are in mammalian species, whose body temperatures and other physiological factors are different from those of avian and insect species. Therefore, when the test is performed on avian or insect cells, the validity of the data is open to question unless a tumorigenic cell line of the species being tested is included as a positive control. The NRA/NCL may accept the results of an in vitro test, such as growth in soft agar, as a substitute for the in vivo test for avian and insect cell lines. However, as mentioned above, correlations of in vitro tests with in vivo tests are imperfect. This should be discussed with the NRA/NCL.

Many CCLs (e.g. BHK-21, CHO, HeLa) are classified as tumorigenic because they possess the capacity to form tumours in immunosuppressed animals such as rodents. Some CCLs become tumorigenic at high PDLs (e.g. Vero), although they do not possess this capacity at the lower PDLs at which vaccine manufacture occurs. A critical feature regarding the pluripotency of embryonic SCLs, even though they display a diploid karyotype, is that they form tumours in immunocompromised mice.

The expression of a tumorigenic phenotype can vary from one CCL to another, and even within different sublines of the same CCL. This range of variability, from non-tumorigenic, to weakly tumorigenic, to highly tumorigenic, has been viewed by some as indicating different degrees of risk when the CCLs are used as substrates for the manufacture of biological products (10, 11).

If the CCL has already been demonstrated to be tumorigenic (e.g. BHK-21, CHO, HEK293, Cl27), or if the class of cells to which it belongs is tumorigenic (e.g. hybridomas, SCLs), it may not be necessary to perform additional tumorigenicity tests on cells used for the manufacture of therapeutic products. Such cell lines may be used as cell substrates for the production of biologicals if the NRA/NCL has determined, on the basis of characterization data and manufacturing data, that issues of purity, safety and consistency have been addressed. A new cell line (DCL, SCL or CCL) should be presumed to be tumorigenic unless data
demonstrate that it is not. If a manufacturer proposes to characterize the cell line as non-tumorigenic, the following tests should be undertaken.

Cells from the MCB or WCB propagated to the proposed in vitro cell age used for production, or beyond, should be examined for tumorigenicity, in a test approved by the NRA/NCL. The test should involve a comparison between the cell line and a suitable positive reference preparation (e.g. HeLa cells) and a standardized procedure for evaluating results.

B.8.2 Type of test animals

A variety of animal systems have been used to assess the tumorigenic potential of cell lines. Table A3.4 lists several examples of such tests, along with advantages and disadvantages of each. Because assessing the tumorigenic phenotype of a cell substrate requires the inoculation of xenogeneic or allogeneic cells, the test animal should be rendered deficient in cytotoxic T-lymphocyte (CTL) activity. This can be accomplished either by the use of rodents that are genetically immunocompromised (e.g. nude mice, severe combined immunodeficiency (SCID) mice) or by inactivating the T-cell function with antithymocyte globulin (ATG), antithymocyte serum (ATS) or antilymphocyte serum (ALS). The use of animals with additional defects in natural killer (NK)-cell function – such as the non-obese diabetic (NOD)-SCID mouse, the NOD-SCID-gamma mouse and the CD3 epsilon mouse – has not yet been explored for cell-substrate evaluation, but they may offer some advantages. In addition to these systems, several other in vivo systems such as the hamster cheek pouch (HCP) model and ATG-treated non-human primates have been used in the past, but are rarely used at present.

Table A3.4

<table>
<thead>
<tr>
<th>Test and brief description</th>
<th>Advantage(s)</th>
<th>Disadvantage(s)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult athymic mouse (Nu/Nu genotype): animals inoculated by the intramuscular or subcutaneous routes with cells to be tested</td>
<td>• Animals readily available • No immunosuppression required</td>
<td>• Higher frequency of spontaneous tumours than in other animal models that are not genetically immunosuppressed • Low sensitivity for assessing the metastatic potential of the inoculated cells</td>
<td>(65)</td>
</tr>
</tbody>
</table>
# Table A3.4 continued

<table>
<thead>
<tr>
<th>Test and brief description</th>
<th>Advantage(s)</th>
<th>Disadvantage(s)</th>
<th>References</th>
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</table>
| **Newborn athymic mouse:** animals inoculated by the subcutaneous route with cells to be tested | • No immuno-suppression required  
• More sensitive than adults | • Low sensitivity for assessing the metastatic potential of the inoculated cells  
• Since litters include heterozygous mice, twice the number of animals must be inoculated in order to be sure that a sufficient number of homozygous mice have been included.  
• Cannibalism of neonates by the mother | (66) |
| **SCID mouse:** animals receive subcutaneous, intradermal or intrakidney capsule inoculation of test cells | • No immuno-suppression required  
• Potentially increased sensitivity  
• Animals readily available | • Highly susceptible to viral, bacterial and fungal infections  
• Infections can affect the results and reproducibility of studies  
• Spontaneous thymic lymphomas may occur | (66, 67) |
| **Newborn rat:** animals immunosuppressed with ATG, followed by intramuscular or subcutaneous inoculation of cells to be tested | • Animals readily available  
• Sensitive model for detecting metastases  
• Very low frequency of spontaneous tumour formation | • Standardized ATG not available as a commercial product  
• Careful qualification and characterization of the ATG is required to find the balance between immunosuppressive capacity and toxicity | (65, 68) |
| **Newborn hamster or mouse:** animals immunosuppressed with ATS, followed by intramuscular or subcutaneous inoculation of cells to be tested | • Animals readily available | • Cannibalism of neonates by the mother  
• Standardized ATS not available and difficult to balance toxicity versus immunosuppressive capacity | (69) |
### Table A3.4 continued

<table>
<thead>
<tr>
<th>Test and brief description</th>
<th>Advantage(s)</th>
<th>Disadvantage(s)</th>
<th>References</th>
</tr>
</thead>
</table>
| *Newborn hamster or mouse:* animals immunosuppressed with ALS, followed by intramuscular or subcutaneous inoculation of cells to be tested | • Increased sensitivity compared to the HCP test  
• Animals readily available | • Cannibalism of neonates by the mother  
• Standardized ALS not available and difficult to balance toxicity versus immunosuppressive capacity | (70) |
| *HCP:* animals immunosuppressed with cortisone, followed by inoculation of the cells to be tested into the cheek pouch | • Animals readily available | • Lower sensitivity than newer models | (71) |
| *Non-human primates:* animals immunosuppressed with ATG, followed by inoculation of cells to be tested into the muscle of the four limbs | • Species closer to human | • Standardized ATG not available  
• Animals not readily available  
• Expense and limited availability preclude using large numbers  
• Animal welfare principles mandate against use of non-human primates if the same results can be obtained from lower species | (72) |

Although all the animal models listed in Table A3.4 have been used to assess the tumorigenicity of cells, several sensitivity parameters from studies using positive-control cells should be considered when attempting to compare the various in vivo tumorigenicity models. These sensitivity parameters are:

(i) frequency of tumour formation;
(ii) time to appearance of tumours;
(iii) size of tumours;
Factors (i), (ii), (iii) and (v) usually depend on the number of cells inoculated (i.e. they are dose dependent). In addition, the rate of spontaneous tumour formation should be considered. Although comparisons of two or more assays have been reported in the literature (68, 73, 74) none take all of these factors into account, nor do they use the same tumorigenic cell lines. Thus, it is not possible to draw definitive conclusions about the relative sensitivity of the various tumorigenicity assays. Nevertheless, the following points appear to be generally accepted:

- the ATS-treated newborn rat and the ATG-treated non-human-primate systems are the most sensitive for assessing the metastatic potential of inoculated cells;
- ATS and ATG provide better immunosuppression than ALS;
- the nude mouse has a better-defined level of immunosuppression than models that depend on ALS, ATS or ATG, and interlaboratory comparisons of data from nude mice are more likely to yield valid conclusions.

Overall experience during the past 30 years, taking into consideration the points mentioned above, has led to the conclusion that the athymic nude mouse is an appropriate test system for determining the tumorigenic potential of cells proposed for use in the production of biologicals. The major advantages of the athymic nude mouse system are that it is easier to establish and standardize and is generally available, while the newborn rat system is more sensitive for assessing the metastatic potential of tumorigenic cells. In some cases, it may be preferable to use newborn athymic nude mice, as these animals are more sensitive than adults for the detection of weakly tumorigenic cells (66). A tumorigenicity testing protocol using athymic nude mice is provided as Appendix 2. The animal system selected should be approved by the NRA/NCL.

B.8.3 The point in cells’ life history at which they should be tested

Investigation of tumorigenicity should form part of the early evaluation of a new cell substrate for use in production. Cells from the MCB or WCB, propagated to the proposed in vitro cell age and used for production or beyond should be examined for tumorigenicity. The extra population doublings (e.g. 3–10) ensure that the results of the tumorigenicity test can be used in the assessment of overall safety of the product, even assuming a worst-case situation, and this therefore provides a safety buffer.
B.8.4  **Use of control cells**

The tumorigenicity test should include a comparison between the CCL and a positive-control reference preparation such as HeLa cells from a reliable source. This source is preferred in order to standardize the test between laboratories, so that cumulative experience over time can be assessed and made available to NRAs/NCLs and manufacturers, to assist them in the interpretation of data. However, other sources for establishing positive-control cells may be acceptable. The purpose of the positive control is to assure that an individual test is valid, by demonstrating that the animal model has the capacity to develop tumours from inoculated cells (i.e. a negative result is unlikely to be due to a problem with the in vivo model). If the positive-control cells fail to develop tumours at the expected frequency, then this could be indicative of problems (such as infections) in the animals or in the testing facility, which can reduce the efficiency of tumour development.

When the cell substrate has been adapted to growth in serum-free medium, which may contain growth factors and other components that could influence growth as well as detection of a tumorigenic phenotype, consideration should be given to processing the positive-control cells in the same medium. Whenever possible, both the test article and the positive-control cells should be resuspended in the same medium, such as phosphate-buffered saline (PBS), for inoculation.

In designing a tumorigenicity protocol, it is important to recognize that tumours arise spontaneously in nude mice and that the incidence of such tumours increases with the age of the mice. Therefore, databases (both published data and the unpublished records/data of the animal production facility that supplied the test animals) of rates of spontaneous neoplastic diseases in nude mice should be taken into account during the assessment of the results of a tumorigenicity test. In general, negative controls are not recommended because the rates of spontaneous neoplastic disease in nude mice are low, and small numbers of negative-control animals are unlikely to provide meaningful data. However, if negative-control cells such as WI-38, MRC-5 or FRhl-2 are included, clear justification for including them should be provided. For example, if serum-free medium is used to grow the cell substrate, it is conceivable that growth factors may influence the appearance of spontaneous tumours; consequently, negative-control cells suspended in the same medium may be needed to interpret the test results.

B.8.5  **Number of test animals**

To determine whether the cells being characterized have the capacity to form tumours in animals, the cells being tested, the reference positive-control cells and, if any, the reference negative-control cells should be injected into separate groups of 10 animals each. In a valid test, progressively growing tumours should be produced in at least 9 out of 10 animals injected with the positive reference cells.
B.8.6 **Number of inoculated cells**

Each animal should be inoculated intramuscularly or subcutaneously (75) with a minimum of $10^7$ viable cells. If there is no evidence of a progressively growing nodule at the end of the observation period, the cell line may be considered to be non-tumorigenic. If the cell line is found to be tumorigenic, the NRA/NCL may request additional studies to determine the level of tumorigenicity. This can be done with dose–response studies, where doses of $10^7$, $10^5$, $10^3$ and $10^1$ viable cells are inoculated, and the data can be expressed as tumour-producing dose at the 50% end-point (TPD$_{50}$ value) (76).

B.8.7 **Observation period**

Animals are examined weekly by observation and palpation for evidence of nodule formation at the site of injection. The minimum observation period depends on the test system selected. In the case of the nude mouse, a minimum of 4 months is recommended. A shorter period is recommended for the ATS-treated newborn rat because the immunosuppressive effect of the ATS declines after the final injection at 2 weeks.

B.8.8 **Assessment of the inoculation site over time (progressive or regressive growth)**

If nodules appear, they are measured in two perpendicular dimensions, the measurements being recorded weekly to determine whether the nodule grows progressively, remains stable or decreases in size over time. Animals bearing nodules that are progressing should be killed before the end of the study if the tumour reaches the limit set by the relevant authorities for the humane treatment of animals. Animals bearing nodules that appear to be regressing should not be killed until the end of the observation period. Cell lines that produce nodules that fail to grow progressively are not considered to be tumorigenic. If a nodule persists during the observation period and retains the histopathological characteristics of a tumour, this should be investigated further and discussed with the NRA/NCL.

B.8.9 **Final assessment of the inoculation site**

At the end of the observation period, all animals, including the reference group(s), are killed and examined for gross and microscopic evidence of the growth of inoculated cells at the site of injection and other sites.

B.8.10 **Evaluation of animals for metastases**

Animals are examined for microscopic evidence of metastatic lesions in sites such as the liver, heart, lungs, spleen and regional lymph nodes.
B.8.11 **Assessment of metastases (if any)**
Any metastatic lesions are examined further to establish their relationship to the primary tumour at the injection site. If what appears to be a metastatic tumour differs histopathologically from the primary tumour, it is necessary to consider the possibility that this tumour either developed spontaneously or was induced by one or more of the components of the cell substrate, such as an oncogenic virus. This may require further testing of the tumour itself, or the tumorigenicity assay may need to be repeated. In such cases, appropriate follow-up studies should be discussed and agreed with the NRA/NCL (also see section B.9, “Oncogenicity”).

B.8.12 **Interpretation of results**
A CCL is considered to be tumorigenic if at least 2 out of 10 animals develop tumours at the site of inoculation within the observation period. However, the reported rate of spontaneous neoplastic diseases in the test animals should be taken into account during the assessment of the results. In addition, the histopathology of the tumours must be consistent with the inoculated cells, and a genotypic marker should show that the tumour is not of nude mouse origin.

If only one of 10 animals develops a tumour, it is appropriate to investigate further in order to determine, for example, if the tumour originated from the cell substrate inoculum or from the host animal and whether there are any viral or inoculated cell DNA sequences present. The NRA/NCL should be consulted in this regard.

The dose–response of the CCL may be studied in a titration of the inoculum as part of the characterization of the CCL. The need for such data will depend on many factors specific to a given CCL and to the product being developed. The NRA/NCL should be consulted in this regard.

**Applicability**
- **Cell banks:** representative EOPC or ECB from the MCB or first WBC
- **Cell types:** DCL, SCL, CCL

B.9 **Oncogenicity**

**B.9.1 Tests for oncogenicity**
While tumorigenicity is the property of cells to form tumours when inoculated into susceptible animals, oncogenicity is the property of an acellular agent to induce cells of an animal to become tumour cells. As such, tumours that arise in a tumorigenicity assay contain cells derived from the inoculated cells, while tumours that arise in an oncogenicity assay are derived from the host. Oncogenic activity from cell substrates could be due either to the cell substrate DNA (and
perhaps other cellular components) or to an oncogenic viral agent present in
the cells. Although there may be a perception that the cellular DNA from highly
tumorigenic cells would have more oncogenic activity than the DNA of weakly or
non-tumorigenic cells, it is not currently known if there is a relationship between
the tumorigenicity of a cell and the oncogenicity of its DNA. Nevertheless, the
NRA/NCL may require oncogenicity testing of the DNA and cell lysate from a
new cell line (i.e. other than those such as CHO, NS0, Sp2/0 and low-passage Vero,
for which there is considerable experience) that is tumorigenic in animal model
systems (see below), because of the perception that a vaccine manufactured in
such a cell line poses a neoplastic risk to vaccine recipients.

The major complication in assaying cellular DNA in animals arises from
the size of the mammalian genome. Because the mammalian haploid genome is
approximately \(3 \times 10^9\) base pairs (bp), whereas the size of a typical oncogene could
be \(3-30 \times 10^3\) bp, the concentration of an oncogene in cellular DNA expression
systems would be about \(10^5-10^6\)-fold less concentrated than a plasmid containing
the same oncogene. As a consequence, if 1 \(\mu\)g of an oncogene expression plasmid
induces a tumour in an experimental animal model, the amount of cellular DNA
that would contain a similar amount of the same oncogene is \(10^5-10^6\) \(\mu\)g (i.e.
100 mg to 1 g). To date, three studies have indicated that between 1 and 10 \(\mu\)g
of expression plasmids for cellular oncogenes can be oncogenic in mice (34, 43,
44). Therefore, more sensitive in vivo assays need to be developed before the
testing of the oncogenic activity of cellular DNA becomes practicable. Recent
results suggest that the sensitivity of the assay can be increased by several orders
of magnitude, with the use of certain immune-compromised strains of mice that
are prone to develop tumours after inoculation with oncogenes. Thus, it may be
possible to assess the oncogenic activity of cellular DNA in the future. However,
at present there is no standardized in vivo oncogenicity test for cellular DNA.
An example of a protocol is nonetheless provided in Appendix 3.

Several in vitro systems, such as scoring the neoplastic transformation of
NIH 3T3 cells in a focus-forming assay following transfection of oncogenic DNA
(77–79), have been used to assess oncogenicity. However, it is not clear how such
assays reflect the oncogenic activity of DNA in vivo, since they predominantly
detect the oncogenic activity of activated ras-family members, and thus it is
unclear how the assays can assist in estimating risk associated with the DNA or
cell lysate from a cell substrate.

Based on experience with DCLs WI-38, MRC-5 and FRhL-2, testing of
new MCBs of these cell lines for oncogenicity is not recommended. Other DCLs
for which there is substantial experience may also not need to be tested. The NRA/
NCL should be consulted in this regard. As stated in section B.8.1, a new CCL
should be presumed to be tumorigenic unless data demonstrate that it is not. If
a manufacturer demonstrates that a new CCL is non-tumorigenic, oncogenicity
testing on cell DNA and cell lysates might not be required by the NRA/NCL.
When appropriate, and particularly for vaccines, cell DNA and cell lysates should be examined for oncogenicity in a test approved by the NRA/NCL. An oncogenicity testing protocol is provided as Appendix 3.

**Applicability**

- **Cell banks**: MCB or first WCB taken to representative EOPC or ECB
- **Cell types**: CCL, SCL (recommended when tumorigenic cells are used in vaccine production)

**B.10 Cytogenetics**

**B.10.1 Characterization**

Chromosomal characterization and monitoring were introduced in the 1960s, to support the safety and acceptability of human DCLs as substrates for vaccine production. Human DCLs differ from CCLs by retaining the characteristics of normal cells, including the normal human diploid karyotype. A significant quantity of data has been accumulated since then, and this has led to the conclusion that less extensive cytogenetic characterization is appropriate because of the demonstrated karyotypic stability of human DCLs used in vaccine production (80). Thus, the use of karyology as a lot-by-lot quality-control test is unnecessary for well-characterized and unmodified human DCLs (e.g. WI-38, MRC-5) and for FRhL-2.

Cytogenetic data may be useful for the characterization of CCLs, especially when marker chromosome(s) are identified. Such data may be helpful in assessing the genetic stability of the cell line as it is expanded from the MCB to the WCB and finally to production cultures (see section B.3). The following recommendations are appropriate for the characterization of DCL and CCL cell banks.

Cytogenetic recharacterization of DCLs (e.g. WI-38, MRC-5 and FRhL-2) should not be required unless the cells have been genetically modified or the culture conditions have been changed significantly, since such data are already available (19–21). However, for each WCB generated, manufacturers should confirm once that the cells grown in the manner to be used in production are diploid and have the expected lifespan.

To determine the general character of a new or previously uncharacterized DCL, samples from the MCB should be examined at approximately four equally spaced intervals during serial cultivation from the MCB through to the proposed in vitro cell age used for production or beyond. The testing intervals should be agreed with the NRA. Each sample should consist of a minimum of 100 cells in metaphase and should be examined for exact counts of chromosomes as well as for breaks and other structural abnormalities.
Giemsa-banded karyotypes of an additional five metaphase cells in each of the four samples may provide additional useful information. The ISCN (81) 400 band is the minimum acceptable level of Giemsa-banding analysis for human cells.

Stained slide preparations of the chromosomal characterization of the cells (i.e. DCL, CCL), or photographs of these, should be maintained permanently as part of the cell-line record. Further recommendations have been proposed for SCLs (57).

**Applicability**

- **Cell banks**: MCB, ECB or representative EOPC
- **Cell types**: DCL, SCL, CCL (as a test for genetic stability, when appropriate)

**B.11 Microbial agents**

**B.11.1 General considerations**

While many biological production systems require human or animal cell substrates, such cells are subject to contamination with, and have the capacity to propagate, extraneous, inadvertent or so-called adventitious organisms such as mycoplasma and viral agents. In addition, animal cells contain endogenous agents such as retroviruses that may also be of concern. Testing for both endogenous (e.g. retroviruses) and adventitious agents (e.g. mycoplasmas) is described in the subsequent sections. In general, cell substrates contaminated with microbial agents are not suitable for the production of biologicals. However, there are exceptions to this general rule. For example, the CHO and other rodent cell lines that are used for the production of highly purified recombinant proteins express endogenous retroviral particles. The balance of risk versus benefit must be considered when determining the suitability of a cell substrate for the production of a specific product. Further, risk-mitigation strategies during production, including purification (removal) and inactivation by physical, enzymatic and/or chemical means, should be implemented whenever appropriate and feasible. Even though a cell substrate might be unacceptable for some products, such as a live viral vaccine subjected to neither significant purification nor inactivation, that same cell substrate may be an acceptable choice for a different type of product, such as a highly purified recombinant protein or monoclonal antibody for which risk mitigation has been achieved by significant and validated viral clearance in the production process.

A strategy for testing cell banks for microbial agents should be developed. One strategy is to perform exhaustive testing at the MCB level and to carry out
more limited testing on the WCB derived from the MCB. This more limited testing would be selected on the basis of those agents that could potentially be introduced during the production of the WCB from the MCB. Testing would not need to be replicated for agents that could only have been present prior to the production of the MCB (e.g. an endogenous retrovirus, or BVDV from serum used for developing the cell seed or in the legacy of establishing the cell line).

However, if the number of vials of an MCB is limited, an alternative strategy would be to conduct the more exhaustive testing on the first WCB made from that MCB, and to limit testing on the MCB itself. An advantage to the strategy of performing more exhaustive testing on the first WCB is that it provides a greater opportunity for amplification of any agents that may have been introduced earlier and through to production of the WCBs. There are advantages and disadvantages to more extensive testing of the MCB or the WCB, and consideration should be given to what is more appropriate for the particular product(s) to be manufactured using a given cell bank. Consultation with the NRA/NCL should be considered prior to implementation, to determine whether a proposed testing strategy is acceptable.

EOPC/ECB should be characterized once for each commercial production process. Testing of the ECB serves as further characterization of the MCB or WCB that was exhaustively tested. It also permits additional time/passages for amplification of low-level contaminants or reactivation of viral contaminants that may have been missed in the testing of the upstream bank.

B.11.2 Viruses

Manifestations of viral infections in cell cultures vary widely among the broad array of virus families that are potential contaminants; thus, the methods used to detect them vary. Lytic infections are frequently detected by the CPE they cause. However, in some cases such as non-cytopathic BVDV, no CPE is observed. Viruses also may be present latently (e.g. herpesviruses) or endogenously (in the germline, e.g. retroviral proviruses). Specific techniques such as molecular and immunological methods and electron microscopy may be required to reveal the presence of such inapparent infections. For new cell substrates, induction of a detectable infection by exposing the cells to special conditions (e.g. chemical induction, heat shock) may be required, and special detection techniques such as transcriptome sequencing or degenerate primer PCR may be useful.

The strategy developed to test cell substrates for viruses should take into consideration the families of viruses and specific viruses that may be present in the cell substrate. Consideration should be given to the species and tissue source from which the cell substrate originated, and to the original donor’s medical history in the case of human-derived cell substrates or to the pathogen status of donor animals in the case of animal-derived cell substrates. Consideration should
also be given to viruses that could contaminate the cell substrate from the donors or from animal- or human-derived raw materials used in the establishment and passage history (legacy) of the cell substrate prior to and during cell banking or production (e.g. serum, trypsin, animal- or human-derived medium components, antibodies used for selection, or animal species through which the cell substrate may have been propagated), as well as laboratory contamination from operators or other cell cultures.

Tests should be undertaken to detect, and where possible identify, any endogenous or exogenous agents that may be present in the cells. Attention should be given to tests for agents known to cause an inapparent infection in the species from which the cells were derived, thereby making it more difficult to detect (e.g. simian virus 40 (SV-40) in rhesus monkeys).

Primary cells are obtained directly from the tissues of healthy animals and are more likely to contain adventitious agents than banked, well-characterized cells. In addition, recent vaccination of source animals should be considered, as the animals may be exposed to live vaccines. The risk with primary cells can be mitigated by rigorous qualification of source animals and of the primary cells themselves. When feasible, animals from which primary cultures are established should be from genetically closed flocks, herds or colonies that are monitored for freedom from pathogens of specific concern. Such animals are known as specific-pathogen-free, or SPF. The term “closed” refers to the maintenance of a group (flock, herd or colony) free from introduction of new animals (new genetic material that could introduce new retroviral proviruses, for instance). Many live viral vaccines are commonly produced in primary cells and undergo little purification during production. In such cases, and when feasible, the use of SPF animals is highly recommended. Documentation of the status of the source animals should be provided to the NRA/NCL. Animals that are not from closed flocks, herds or colonies should be quarantined and thoroughly evaluated for a period that is sufficient to detect signs of disease or infection (e.g. monkeys are generally quarantined for six or more weeks (82)). Such animals also should be screened serologically for appropriate adventitious agents, in order to determine their suitability as a source for the primary cell substrate. Animal husbandry practices should be documented. Even so, viral contamination of the cells may not be excluded from all cultures. For example, contamination of primary monkey kidney cells with foamy virus or simian cytomegalovirus is common in the absence of specific concerted efforts to prevent such contaminations.

For primary cell cultures, the principles and procedures outlined in Part C of Recommendations for the production and control of poliomyelitis vaccine (oral) (82), together with those in section A.4 of Requirements for measles, mumps and rubella vaccines and combined vaccine (live) (83) may be followed.

The production of viral vaccines, such as those against smallpox or rabies, originally required the use of living animals, and the great range of possible viral
contaminants became apparent only as cell culture methods were developed. For example, human enteroviruses were not recognized until the development of monkey kidney cell cultures in which they could produce CPEs, because the disease produced in humans is either relatively mild or in some cases non-existent. It was also clear that viruses could be detected in some systems but not others. For instance, the polyomavirus SV40 does not produce a CPE in cultures from rhesus monkey kidney cells (in which much of the early polio vaccines were produced) derived from SV40-infected monkeys but will do so in cultures from cynomolgus or African green monkey kidney cells. The suspicion was, therefore, that there were many viruses in the culture systems of the time and that they were detected only if the assays were appropriate. This remains an accurate view and has led to a range of different approaches for trying to detect all contaminants.

Coxsackie viruses are named after the town in New York where they were first identified after being detected because of their effects in mice. Coxsackie B viruses produce clinical signs and death in adult mice, while Coxsackie A viruses will affect only suckling mice. For many years, tissue culture methods were a less reliable method of detection of Coxsackie A viruses than suckling mice, and the continued use of these animals in cell bank characterization reflects this.

In the 1940s, embryonated chicken eggs were a popular substrate for the growth and assay of viruses such as influenza, measles, mumps, yellow fever and vaccinia. They therefore appear to have a wide range of susceptibility. Simian viruses such as SV5 or viruses such as Sendai virus also grow well in them. As many are paramyxoviruses with haemagglutinating activity, the egg-based assays include tests for haemagglutinating activity, as well as for the death of the embryos.

A range of tissue culture cells is also used, typically including one human, one of the same species as the production cell, and one other (often of monkey origin). The hope is that the range will catch viruses not detected by other means, although in practice it is wise to assume that there is no such thing as a generic detection method; for example, cells from an inappropriate monkey species will not necessarily detect SV40, whereas cells from other species will (e.g. Vero cells). In certain circumstances where a virus is of particular concern, specific tests have been applied. For example, herpes B virus is a common infection in monkeys in the absence of precautions such as quarantine and clinical evaluation of the donor animals, and this has very serious effects on infected humans. While herpes B virus was routinely detected by the use of primary rabbit kidney cell cultures, established rabbit cell lines are now acceptable for this purpose. Another example is Marburg virus, which in the 1970s caused a number of deaths in workers who handled monkeys that were to be used in a vaccine production facility. The incident might have been avoided if the animals had been adequately quarantined. A specific test in guinea-pigs was introduced and maintained for a number of years to ensure the absence of the agent.
There is a disparate range of tests that have been or still are used with the aim of detecting any significant contaminant that may be present in cell cultures. Some, such as the rabbit kidney cell test, are very specific in intent, while others may be expected to be more generic. In general, however, it is wise to assume that an assay will never be all-encompassing, whether based on historical virological approaches or more current methodologies. Thus, the consequences of deleting tests on the grounds of redundancy must be very carefully evaluated before any action is taken. On the other hand, it is difficult to justify the maintenance of a test if it detects only viruses that are also detected by other methods of equivalent sensitivity and comparable ease of use and cost. Each of these considerations should be borne in mind when developing an appropriate testing strategy for the given cell bank. Policies to minimize the use of animals in safety testing should also be considered, but must be balanced with the utility and necessity (sensitivity and ability to detect particular adventitious agents not readily detected by other means) of the test in which they are used.

B.11.2.1 Tests in animals and eggs
The cells of the MCB and WCB are unsuitable for production if any of the animal or egg tests show evidence of the presence of any viral agent attributable to the cell banks.

In general, MCBs are thoroughly characterized by the methods listed below. WCBs may be characterized by an abbreviated strategy, when appropriate. However, an alternative strategy to this general rule may be used, as discussed in section B.11.2 above. These tests may be performed directly on cells, supernatant fluids or cell lysates from the bank itself, or on cells or supernatant fluids or cell lysates from cells from the bank that have been passaged to the proposed in vitro cell age for production or beyond.

In some countries, policies exist to minimize the use of animals in safety testing.

B.11.2.1.1 Adult mice
The original purpose of this test was to detect lymphocytic choriomeningitis virus (LCMV). The test in adult mice for pathogenic viruses includes inoculation by the intraperitoneal route (0.5 ml) with cells and culture fluids from the MCB or WCB, where at least $10^7$ viable cells or the equivalent cell lysate are divided equally among at least 10 adult mice weighing 15–20 g.

In some countries, the adult mice are also inoculated by the intracerebral route (0.03 ml).

In some countries, at least 20 mice are required for each test.
The animals are observed for at least 4 weeks. Any animals that are sick or that show any abnormality are investigated to establish the cause. Animals that do not survive the observation period should be examined for gross pathology and histopathology, in order to determine a cause of death and, if a viral infection is indicated, efforts should be undertaken to identify the virus. Viral identification may involve culture and/or molecular methods. Further, each mouse that dies after the first 24 hours of the test, or is killed because of illness, should be necropsied and examined for evidence of viral infection by subinoculation of appropriate tissue into at least five additional mice, which should be observed for 21 days. The test is not valid if more than 20% of the animals in either the test group or the negative control group (if used), or in both, become sick for nonspecific reasons and do not survive the observation period.

In some countries, the adult mice are observed for 21 days.

If the cell substrate is of rodent origin, at least $10^6$ viable cells or the equivalent cell lysate are injected intracerebrally into each of 10 susceptible adult mice to test for the presence of LCMV.

In some countries, after the observation period, the animals are challenged with live LCMV to reveal the development of immunity against non-pathogenic LCMV contaminants resulting in otherwise inapparent infection.

**Applicability**

- **Cell banks**: MCB, WCB or ECB or representative EOPC
- **Cell types**: PCC, DCL, SCL, CCL

**B.11.2.1.2 Suckling mice**

The original purpose of this test was to detect Coxsackie viruses. The test for pathogenic viruses in suckling mice includes inoculation by the intraperitoneal route (0.1 ml) with cells and culture fluids from the MCB or WCB. At least $10^7$ viable cells or the equivalent cell lysate are divided equally between two litters of suckling mice, comprising a total of at least 10 animals less than 24 hours old.

In some countries, the suckling mice are also inoculated by the intracerebral route (0.01 ml).

In some countries, 20 suckling mice are inoculated.

The animals are observed for at least 4 weeks. Any animals that are sick or show any abnormality are investigated to establish the cause. Animals that do not survive the observation period should be examined for gross pathology and
histopathology, in order to determine the cause of death. If a viral infection is indicated, efforts should be undertaken to identify the virus where practicable. Viral identification may involve culture and/or molecular methods. Further, such examination of viral infection should include subinoculation of appropriate tissue suspensions into an additional group of at least five suckling mice, by intracerebral and intraperitoneal routes, and observation daily for 14 days. In the case of suckling mice, it is often observed that those that perish are cannibalized by their mother and this renders determination of cause of death impossible (when they are fully cannibalized and no remains can be recovered). The test is not valid if more than 20% of the animals in either the test group or the negative control group (if used), or in both, do not survive the observation period.

In some countries, the suckling mice may be observed for a period of 14 days, followed by a subpassage involving a blind passage (via intraperitoneal and intracerebral inoculation into at least five additional mice) of a single pool of the emulsified tissue (minus skin and viscera) of all mice surviving the original 14-day test.

**Applicability**

- **Cell banks:** MCB, WCB or ECB or representative EOPC
- **Cell types:** PCC, DCL, SCL, CCL

**B.11.2.1.3 Guinea-pigs**

The original purpose of this test was to detect LCMV and *Mycobacterium tuberculosis*. When it is necessary to detect *Mycobacterium* species, a test in guinea-pigs is performed and includes inoculation by the intraperitoneal route (5 ml) with cells and culture fluids from the MCB or WCB, where at least $10^7$ viable cells or the equivalent cell lysate are divided equally among the animals.

In some countries, five guinea-pigs weighing 350–450 g are also inoculated by the intracerebral route (0.1 ml) and observed for 42 days to reveal *Mycobacterium* tuberculosis and other species.

The animals are observed for at least 6 weeks. Animals that are sick or show any abnormality are investigated to establish the cause. Animals that do not survive the observation period should be examined for gross pathology and histopathology, in order to determine a cause of death and, if a viral infection is indicated, efforts should be undertaken to identify the virus. Viral identification may involve culture and/or molecular methods. The test is not valid if more than 20% of the animals in either the test group or the negative control group (if used), or in both, do not survive the observation period.
The test in guinea-pigs for the presence of *Mycobacterium* may be replaced by an alternative in vitro method such as culture, or shortened culture with a PCR end-point (also see section B.11.3).

**Applicability**

- **Cell banks:** MCB, WCB or ECB or representative EOPC
- **Cell types:** PCC, DCL, SCL, CCL (the latter three are dependent on legacy and current use of media components of animal origin that could result in contamination with mycobacterial species)

**B.11.2.1.4 Rabbits**

The original purpose of this test was to detect herpes B virus. When it is necessary to detect simian herpes B virus, the test in rabbits for pathogenic viruses is performed and includes inoculation by the intradermal (1 ml) and subcutaneous (>2 ml) routes with cells and culture fluids from the MCB or WCB, where at least $10^7$ viable cells or the equivalent cell lysate are divided equally among the animals.

In some countries, five rabbits weighing 1.5–2.5 kg are inoculated by the subcutaneous route, with either 2 ml or between 9 and 19 ml. Consultation with the NRA/NCL regarding acceptable methods should be considered.

The animals are observed for at least 4 weeks. Animals that are sick or show any abnormality are investigated to establish the cause. Animals that do not survive the observation period should be examined for gross pathology and histopathology, in order to determine a cause of death and, if a viral infection is indicated, efforts should be undertaken to identify the virus. Viral identification may involve culture and/or molecular methods. The test is not valid if more than 20% of the animals in either the test group or the negative control group (if used) do not survive the observation period.

The test in rabbits for the presence of herpes B virus is intended for primary simian cultures, and may be replaced by a test in rabbit kidney cell cultures.

**Applicability**

- **Cell banks:** MCB, WCB or ECB or representative EOPC
- **Cell types:** PCC, DCL, SCL, CCL

**B.11.2.1.5 Embryonated chicken eggs**

At least $10^6$ viable cells or the equivalent cell lysate, along with culture fluids from the MCB or WCB of avian origin, propagated to the proposed in vitro cell age for production or beyond, are injected into the allantoic cavity of each of at least
10 embryonated hens’ eggs, and into the yolk sac of each of at least another 10 embryonated hens’ eggs. The eggs are examined after not fewer than 5 days of incubation. The allantoic fluids of the eggs are tested with red cells from guinea-pig and chicken (or other avian species) for the presence of haemagglutinins. The test is not valid if more than 20% of the embryonated hens’ eggs in either the test group or the negative control group (if used), or in both, are discarded for nonspecific reasons.

In some countries, the NRA/NCL also requires that other types of red cells, including cells from humans (blood group IV O) or monkeys, should be used in addition to cells from guinea-pig and chicken (or other avian species). In all tests, readings should be taken after incubation for 30 minutes at 0–4 °C, and again after a further incubation for 30 minutes at 20–25 °C. For the test with monkey red cells, readings also should be taken after a final incubation for 30 minutes at 34–37 °C.

In some countries, inoculation by the amniotic route is used.

In some countries, following incubation, allantoic fluids or a 10% suspension of yolk sacs, as appropriate, should be harvested, pooled and blind passaged into an additional group of eggs.

The eggs used for the yolk-sac test should usually be 5–7 days old. The eggs used for the allantoic cavity test should be 9–11 days old.

Alternative ages for the embryonated chicken eggs and alternative incubation periods are acceptable if they have been determined to be equivalent or better for detecting the presence, in the test samples, of the adventitious agents that the test is capable of detecting when performed as above.

Embryos that do not survive the observation period should be examined for gross pathology, in order to determine a cause of death and, if a viral infection is indicated, efforts should be undertaken to identify the virus. Viral identification may involve culture and/or molecular methods.

**Applicability**

- **Cell banks:** MCB of avian origin, WCB of avian origin or ECB or representative EOPC
- **Cell types:** avian PCC, DCL, SCL, CCL (also recommended for novel cell substrates)

B.11.2.1.6 *Antibody production tests*

Rodent cell lines are tested for species-specific viruses using mouse, rat and hamster antibody production tests, as appropriate. In vivo testing for lymphocytic
choriomeningitis virus, including a challenge for non-lethal strains, is performed for such cell lines, as described in section B.11.2.1.1. Avian cell lines may also be tested using a chicken antibody production test – e.g. to detect chicken anaemia virus. Further, if the cell substrate (even if not of rodent origin) has been exposed to materials of rodent origin (e.g. selection using a monoclonal antibody), testing should be considered for the species-relevant viruses, using an antibody production test (84, 85).

In some countries, consideration is being given to use of nucleic acid testing in place of the in vivo antibody production testing. In these cases, data should be provided to the NRA/NCL to justify this practice.

**Applicability**

- **Cell banks**: MCB, WCB or ECB or representative EOPC
- **Cell types**: DCL, SCL, CCL (recommended primarily for cells of rodent origin)

**B.11.2.2 Tests in cell culture**

Tests in cell culture are capable of detecting a broad array of viral families. Readouts include monitoring the cultures periodically for CPE and tests for haemadsorbing and haemagglutinating viruses, which are conducted at the end of the culture period. In addition to the indicator cells described below, it may be appropriate to expand the different types of indicator cells used (beyond two or three) to enable the detection of viruses with differing host requirements. Decisions about which cell lines to use as indicator cells should be guided by the species and legacy of the production cell substrate, taking into consideration the types of viruses to which the cell substrate could potentially have been exposed and thus the viruses one would like to detect by this assay method. The cell substrate is unsuitable for production if any of the indicator cell cultures shows evidence of the presence of any viral agent attributable to the tested cell substrate.

**Applicability**

- **Cell banks**: MCB, WCB or ECB or representative EOPC
- **Cell types**: PCC, DCL, SCL, CCL

**B.11.2.2.1 Indicator cells**

Live cells or cell lysate, each with spent culture fluids of the MCB or WCB, are inoculated on to monolayer cultures or cultivated with monolayer cultures of the cell types listed below, as appropriate.
A lysate of the cells may be prepared by a method that avoids virus disruption while allowing maximal virus release (e.g. typically three freeze/thaw cycles followed by low-speed centrifugation). If cells, lysate or spent culture fluids are to be stored prior to testing, they should be stored at ≤−70 °C.

Cultures (primary cells or CCL) of the same species and tissue type as that used for production may be used.

Cultures of a human DCL may be used. The original purpose of this test, using primary human cells, was the detection of measles virus. Where the cell substrate is of human origin, a simian kidney cell line should be used as the second indicator cell line. The original purpose of the use of this cell type was the detection of simian viruses.

In some countries, cultures of another (third) cell line from a different species are required.

In many circumstances, more than two cell lines may be necessary to cover the range of potential viral contaminants and, typically, a third cell line of simian origin would be used if the cell substrate is not of simian origin.

For new cell substrates, additional cell lines to detect viruses known to be potentially harmful to humans could be considered (e.g. for insect cell lines; if the cells selected for the above-mentioned tests are not known to be permissive to insect viruses, an additional detector cell line should be included in the testing).

The cell bank sample to be tested is diluted as little as possible. At least $10^7$ cells, or equivalent cell lysate, and spent culture fluids are inoculated on to each of the indicator cell types. The resulting co-cultivated or inoculated cell cultures are observed for evidence of viruses by CPE for at least 2 weeks. If the cell line is known to be capable of supporting the growth of human or simian cytomegalovirus, HDC cultures are observed for at least 4 weeks. Extended (4 week) cell culture for the purposes of detecting human or simian cytomegalovirus can be replaced by the use of NAT to detect cytomegalovirus nucleic acid.

In some countries, a passage on to fresh cultures for an additional 2 weeks is recommended for all indicator cultures. In some cases, it may be difficult to keep the cell cultures healthy for 2 weeks without subculturings. In these cases, it may be necessary to feed the cultures with fresh medium or to subculture after 2 weeks on to fresh cultures, in order to be able to detect viral agents.

At the end of the observation period, samples of each of the co-cultivated or inoculated cell culture systems are tested for haemadsorbing and/or haemagglutinating viruses, as described in section B.11.2.1.5.
B.11.2.2.2 Additional considerations regarding the tests in cell culture for insect viruses

Many insect cell lines carry persistent viral infections that do not routinely produce a noticeable CPE (e.g. some clones of the Hi-5 cell line are persistently infected with an insect nodavirus). However, the viruses may be induced to replicate by stressing the cells with a variety of techniques such as increased/reduced culture temperature (above or below that routinely used for production), heat shock for a short period, superinfection with other insect viruses, or chemical inducers. Therefore, the probability of detecting such low-level persistent infections may be increased by stressing the cells prior to analysis.

Intact cells and cell lysates from a passage level at or beyond that equivalent to the EOPC are co-cultivated with indicator cells from at least three different species of insect in addition to the indicator cells noted in section B.11.2.2.2. Cell lines should be selected on the following basis: one of the lines has been demonstrated to be permissive for the growth of human arboviruses, a second line has been shown to be permissive for the growth of a range of insect viruses, and the third has been derived from a species that is closely related to the host from which the MCB is derived (or another line from the same species). Duplicate cultures of indicator cells are typically incubated at two temperatures – such as 37 ± 1 °C and a lower temperature such as 28 ± 1 °C – observed for a period of 14 days, and examined for possible morphological changes. The cell culture fluids from the end of the test period are tested for haemagglutinating viruses, or the intact cells from the end of the test period are tested for haemadsorbing viruses. The cells comply with the test if no evidence of any viral agent is found.

Several mosquito cell lines are available that are permissive for the growth of some human arboviruses and could be considered for these tests. Alternatively, BHK-21 cells could be considered for this purpose. The most permissive insect cell lines characterized to date have been derived from embryonic Drosophila tissues. While the mosquito and Drosophila cell lines may be suitable for some aspects of the testing, it should be remembered that many insect cell lines are persistently infected with insect viruses that usually produce no obvious CPE. In addition, many insect cells may be infected with mammalian viruses, such as BVDV, that are known to replicate in insect cells. Demonstrating that the indicator cell lines are themselves free from adventitious agents is an important prerequisite to their use in the testing outlined above. Consideration should also be given to risk-mitigation strategies, as discussed above, for highly purified products for which viral clearance can be achieved and validated.

B.11.2.3 Transmission electron microscopy

At least 200 cells from the MCB or WCB and from the ECB are examined by transmission electron microscopy (TEM) for evidence of contamination
with microbial agents. Methods include negative staining and thin section. A discussion of these methods is provided by Bierley et al. (86). In some cases it may be appropriate to examine more cells, as discussed below for insect cell lines. The NRA/NCL should be consulted in this regard. Any unusual or equivocal observations that may be of microbiological significance should be noted and discussed with the NRA/NCL.

TEM can detect viral particles in a cell substrate, including certain endogenous retroviruses. While TEM is fairly insensitive (generally detecting gross contamination, but not necessarily low-level contamination), it is a generic assay that can detect microbial agents of many types.

**Applicability**

- **Cell banks**: MCB, WCB, or ECB or representative EOPC
- **Cell types**: DCL, SCL, CCL

**Additional considerations on TEM for insect cells**

The general screening test outlined above applies to MCBs and WCBs derived from insect cells. In addition, cell lines should be subjected to stress conditions, such as described in section B.11.2.2.3, prior to examination by TEM. Increasing the number of cells examined may also improve the probability of detecting an agent (e.g. errantiviruses and hemiviruses). The maintenance temperatures and treatments used should be agreed with the NRA/NCL, as should the number of sectioned cells to be examined.

**Tests for retroviruses**

All vertebrate and insect cells that have been analysed possess endogenous, genetically acquired retroviral sequences integrated into chromosomal DNA in the form of proviruses. These sequences may be expressed, or may be induced, as mRNA. In some cases, the mRNA is translated into viral protein, and virus particles (virions) are produced. In many cases, these virions are defective for replication (e.g. avian endogenous retrovirus EAV (endogenous avian retrovirus), CHO cell line gamma-retrovirus (87)), whereas in others (e.g. X-MuLV) the retroviruses may be capable of infecting cells of other species, including human cells.

Consideration should also be given to the possibility that cell banks may be infected with non-genetically acquired retroviruses (exogenous retroviruses), either because the donor animal was infected or through laboratory contamination.

It should be noted that infection by retroviruses is not necessarily associated with any CPE on the cells. Therefore screening assays, such as the PERT assay for reverse transcriptase, or TEM may be required to reveal their presence.
The cells of the MCB or WCB are unsuitable for production if the tests for infectious retroviruses, if required, show evidence of the presence of any viral agent attributable to the substrate that cannot be demonstrated to be cleared during processing. Generally, the downstream manufacturing process for products (e.g. monoclonal antibodies) made in cell substrates that produce retroviral particles (e.g. CHO cells) or infectious endogenous retrovirus (i.e. NS0, Sp2/0 cells) is validated to provide adequate viral clearance (14). The margin of viral clearance required should be agreed with the NRA/NCL.

Chick embryo fibroblasts (CEF) contain defective retroviral elements that frequently produce defective particles with reverse transcriptase activity. This has been the subject of many studies and WHO consultations because they are used for production of live viral vaccine. If evidence is presented that the donor flock is free of infectious retroviruses and there is no evidence that the cultures are contaminated with infectious retroviruses, the cultures can be considered acceptable with respect to retrovirus tests.

Rodent cell lines express endogenous retroviruses, and thus infectivity tests should be performed to determine whether these endogenous retroviral particles are infectious.

Cell lines such as CHO, BHK-21, NS0 and Sp2/0 have frequently been used as substrates for drug production, with no reported safety problems related to virus contamination of the products, and may be classified as “well characterized” because the endogenous retrovirus particles have been studied extensively. Furthermore, the total number of retrovirus-like particles present in the harvest is evaluated quantitatively (TEM or quantitative PCR) on a representative number of lots, and retrovirus clearance is demonstrated with significant safety factors. In these situations, testing for infectious retrovirus may be reduced (e.g. test one lot, then discontinue testing, but repeat when there is a significant change in the cell culture process, such as a change in scale). Sponsors are encouraged to consult with the NRA.

**Applicability**

MCBs and cells that have been propagated to the proposed in vitro cell age for production or beyond. Alternatively, this testing could be performed on WCBs.

- **Cell banks:** MCB or WCB, and ECB or representative EOPC
- **Cell types:** DCL, SCL, CCL

B.11.2.4.1 *Reverse transcriptase assay*

Test samples from the MCBs or WCBs propagated to the proposed in vitro cell age for production or beyond are examined for the presence of retroviruses.

Culture supernatants are tested by a highly sensitive, quantitative PCR-based reverse transcriptase (RT) assay or PERT assay (88–91).
RT activity is not specific to retroviruses and may derive from other sources such as retrovirus-like elements that do not encode a complete or infectious genome (92–96) or cellular DNA-dependent DNA polymerases (97, 98). Attempts to reduce the PERT activity associated with cellular DNA-dependent DNA polymerases have been reported (98–100), although no treatment can eliminate all activity. Thus, the results of such highly sensitive assays need to be interpreted with caution. Use of appropriate controls in the assay can assist in this regard. Since RT activity can be associated with the presence of defective retrovirus-like particles, and since polymerases other than RT can result in apparent RT activity, a positive result in an RT assay is not conclusive evidence of the presence of infective retrovirus. Positive results may require further investigation, such as carrying out infectivity assays (see section B.11.2.4.4). It may also be useful to utilize the conventional RT assay in this investigation to determine whether the RT activity is Mg2+ or Mn2+ dependent. Such testing should be agreed in advance with the NRA/NCL.

CEFs and other cells of avian origin are known to express retroviral elements. The appropriateness of this test with such cells should be discussed with the NRA/NCL. For example, it may be appropriate to direct testing strategies to the detection of infectious avian retroviruses, such as avian leukosis viruses and reticuloendotheliosis virus, including serological screening of flocks that are the source of the CEFs. Additionally, it is known that insect cells have retroviral elements that are detected by a PERT assay, and so they too may test positive by this assay.

B.11.2.4.2 PCR or other specific in vitro tests for retroviruses

If the PERT test gives unclear results, or when it is unavailable, it may be appropriate to screen the cell substrate for species-specific retroviruses, by molecular methods such as PCR, immunofluorescence, enzyme-linked immunosorbent assay (ELISA) or other virus-specific detection methods. Molecular methods, such as PCR, may also be used for quantification of retrovirus-like particles in the production harvests, provided that the method is validated accordingly. Consultation with the NRA/NCL regarding the acceptability of this approach is recommended.

B.11.2.4.3 Infectivity test for retroviruses

When the test sample is found to have RT activity, it may be necessary to carry out infectivity assays to assess whether the activity is associated with replicating virus.

Because rodent cells generally express endogenous retroviruses, the infectivity and in vitro host range of such retroviruses should be assessed. Test samples from the MCB or WCB, propagated to the proposed in vitro cell age for production or beyond, should be examined with infectivity assays for the presence of retroviruses. Cells to be used for these assays should be able to support the
replication of a broad range of viruses; this may require the use of cells of various species and cell types. The testing strategy should be agreed with the NRA/NCL.

It is often possible to increase the sensitivity of assays by first inoculating the test material on to cell cultures that can support retroviral growth, in order to amplify any retrovirus contaminant that may be present at low concentrations. For non-murine retroviruses, test cell lines should be selected for their capacity to support the growth of a broad range of retroviruses, including viruses of human and non-human primate origin (101, 102).

For murine retroviruses, it is important to assess whether the cells release infectious retroviruses and, if so, to determine the host range of those viruses. The testing for murine retroviruses can be complex, and the NRA/NCL should be consulted for guidance. Murine and other rodent cell lines (CHO, NS0, Sp2/0), or hybrid cell lines containing a rodent component, should be assumed to be inherently capable of producing infectious retroviruses or non-infectious retrovirus-like particles. In such cases, the clearance (removal and/or inactivation) of such retroviruses during the manufacturing process should be quantified and should provide a level of clearance acceptable to the NRA/NCL.

Any testing proposed by the manufacturer should be agreed with the NRA/NCL.

B.11.2.5 Tests for particular viruses not readily detected by the tests described in sections B.11.2.1–B.11.2.4 and their subsections

Some viruses, such as hepatitis B or C viruses or human papillomaviruses, cannot be detected readily by any of the methods described above because these viruses are not known to grow readily in cell culture, or are restricted to human host range. Some animal viruses (e.g. bovine polyomavirus and porcine circoviruses) are not readily detected by the routine tests previously described. In such circumstances, it may be necessary to include specific assays for such viruses. While broad general tests are preferable for detecting unknown contaminants, some selected viruses may be screened by using specific assays such as molecular techniques (e.g. nucleic acid amplification). Antibody-based techniques such as immunofluorescence assays may also be employed.

Generally, once the MCB, WCB or ECB has been demonstrated to be free of selected viruses, it may not be necessary to test the cells at later stages (e.g. at the production level) if such viruses cannot be introduced readily during culture.

Human cell lines should be screened using appropriate in vitro techniques for specific viruses that are the cause of significant morbidity, for those viruses that might establish latent or persistent infections, and for viruses that may be difficult or impossible to detect by the techniques described in sections B.11.2.1–B.11.2.4 and their subsections. Selection of the viruses to be screened should take into account the tissue source and medical history of the donor, if available, from whom the cell line was derived.
Under circumstances in which the cell origin or medical history of the donor, if available, would suggest their presence, it may be appropriate to perform specific testing for the presence of human herpesviruses, human retroviruses, human papillomaviruses, human hepatitis viruses, human polyomaviruses, or difficult-to-culture types of human adenoviruses.

Consideration should be given to screening insect cell lines for specific viruses that have been reported to contaminate particular cell lines (e.g. nodaviruses) or that may be present persistently in insect cell lines and that are known to be infectious for humans.

**Applicability**

The NRA/NCL should be consulted with regard to the specific pathogens or selected viruses that should be included in the testing strategy, as these will be directed on a case-by-case basis depending on the species and origin of the cell and the medical history of the donor, if available.

- **Cell banks:** MCB, WCB, or ECB or representative EOPC
- **Cell types:** PCC (as needed), DCL, SCL, CCL

**B.11.2.5.1 Nucleic acid detection methods**

Tests for selected viruses are usually performed using nucleic acid amplification and detection methods. PCR can be performed directly on DNA extracted from the cells, or on cell lysates or supernatant fluids by DNA amplification, or on RNA by reverse transcription followed by DNA amplification (RT-PCR). In this manner, both DNA and RNA viruses can be detected, as can the proviral DNA of retroviruses. PCR primers can be directed against variable regions of viral nucleic acids, in order to ensure detection of a specific virus or viral strain, or against conserved regions of viral sequences shared among strains or within a family, in order to increase the opportunity for detecting multiple related viruses. Standard PCR analysis can be coupled with hybridization methods to increase its versatility, sensitivity and specificity. For example, the use of probes to various regions of the amplicon might be useful for identifying the virus strain or family. However, PCR methods have the limitation that viral genes may not be sufficiently conserved among all members of a particular viral family for the genes to be detected even when conserved regions are selected.

New and sensitive molecular methods with broad detection capabilities are being developed. These are not yet in routine use but, as they become widely available and validated, they will play an increasing role in the evaluation of cell substrates. The sensitivity of these methods, as well as their breadth of detection, should be considered when evaluating their applicability. One of the advantages of some of these new methods is that they have the potential to discover new viruses. These new approaches involve either degenerate PCR for whole virus
families or random-priming methods, which do not depend on a known sequence. Analysis of the resulting amplicons has employed sequencing, hybridization to oligonucleotide arrays, and mass spectrometry (103–105). The new generation of massively parallel sequencing (MPS) methods may have particular utility. They can be applied to detect virions following nuclease treatment to remove cellular DNA and unencapsidated genomes. In this mode, MPS has been used to discover new viruses in serum and other tissues and has revealed the contamination of human vaccines by porcine circovirus (103, 106–110). MPS can also be employed to screen cell substrates for both latent and lytic viruses by sequencing the transcriptome. In this mode, enormous quantities of data are generated and robust bioinformatic methods are required to detect viral sequences by either positive selection against viral databases or negative selection to remove cellular sequences (103, 110, 111). Care is required to exclude false “hits” to viruses due to recognition of transduced cellular sequences present in some viral genomes, or due to viral genes like virokines that have a close homology to cellular genes (103, 105, 111).

It is probable that application of methods of this type will be expected or required by regulatory agencies in future. At present the methods have not been evaluated for sensitivity and specificity and should be thought of as powerful investigational tools that can reveal issues that can be explored by more established methods.

B.11.3 Bacteria, fungi, mollicutes and mycobacteria

The most common contaminants of cell culture are non-viral. These can be introduced easily from the environment, materials, personnel, etc. Furthermore, many such organisms multiply rapidly and can be pathogenic for humans. It is also important in risk evaluation for the manufacturer to bear in mind that standard compendial tests for “sterility” are intended to give an indication of the effectiveness of aseptic processing in preventing general bacterial or fungal contamination and are not capable of isolating all potential bacterial and fungal contaminants. The manufacturer should consult with the NRA/NCL regarding any particular materials or environments where there may be an elevated hazard of contamination with particular types of fastidious organisms.

Biological starting materials, like cell substrates, should be characterized to ensure that they are free of adventitious infectious organisms such as bacteria, fungi, cultivable and non-cultivable mycoplasmas, spiroplasmas (in the case of insect cells or cells exposed to plant-derived materials) and mycobacteria. For a substance to be considered free of such contaminants, the assays should demonstrate, at a predefined level of sensitivity, that a certain quantity of the substance does not contain detectable levels of the contaminant. Testing should be conducted in an aseptic environment under appropriate clean-room conditions, to avoid false-positive results. Testing should include a plan to allow for repeat
testing to deal with potentially false-positive results and a prequalification plan for reagents used in the tests.

Mycobacterial testing may be applied to cell-bank characterization if the cells are susceptible to infection with *Mycobacterium tuberculosis* or other species. Such testing should also be performed on primary cell cultures. It may be necessary to lyse the host cells in order to detect mycobacteria, because some strains may be primarily intracellular.

Detection of mycoplasma or spiroplasma may require different growth conditions from methods used for mammalian cells, although at least one – spiroplasma – can be cultivated at 30 °C. Positive controls for these tests (particularly for spiroplasmas) are an issue that needs to be resolved. Spiroplasmas have been reported as infectious agents in a number of insect species, and insect cell lines have also been reported to cause pathogenic effects in mammals.

**B.11.3.1 Bacterial and fungal sterility**

Tests are performed as specified in Part A, section 5.2 of the Requirements for biological substances no.6 (112) by a method approved by the NRA/NCL. Additional information can be found in national pharmacopoeias and ICH documents (10, 113–115). For the MCB and WCB, the test is carried out using for each medium 10 ml of supernatant fluid from cell cultures. In addition, the test is carried out on at least 1% of the filled containers (i.e. cryopreservation vials) with a minimum of two containers. For supernatant fluid, it is recommended to use the membrane filtration method. For cell bank vial testing, it may be necessary to use the direct inoculation method. Bacteriostasis and fungistasis should be excluded.

**Applicability**

- **Cell banks:** MCB and each WCB
- **Cell types:** PCC, DCL, SCL, CCL

**B.11.3.2 Mollicutes**

Mollicutes are distinguished by an absence of a cell wall and include mycoplasmas, acholeplasmas, spiroplasmas and others. They are parasites of various animals and plants, living on or in the host’s cells. Mollicutes are also a frequent contaminant of cell cultures. In addition to their potential pathogenicity, mycoplasmas compete for nutrients, induce chromosomal abnormalities, interrupt metabolism and inhibit cell fusion of host cells. *M. pneumoniae* is pathogenic for humans, although there are no reported cases of human infections with this organism arising from exposure to cell cultures or cell-derived products. In any case, cell banks should be demonstrated to be free of such contamination, in order to be suitable for the production of biologicals.
B.11.3.2.1 **Mycoplasma and acholesplasma**

Tests for mycoplasmas are performed as specified in Part A, sections 5.2 and 5.3 of the Requirements for biological substances no. 6 (116), or by a method approved by the NRA/NCL. Both the culture method and the indicator cell-culture method should be used. NAT alone, in combination with cell culture or with an appropriate detection method, may be used as an alternative to one or both of the other methods, after suitable validation and discussion with the NRA/NCL. In this case, a comparability study should be carried out. The comparability study should include a comparison of the respective detection limits of the alternative method and official methods. Specificity (mycoplasma panel detected, potential false-positive results due to cross-reaction to other bacteria) should also be considered. More details are available in the *European pharmacopoeia* Chapter 2.6.7 (117). One or more containers of the MCB and each WCB are used for the test.

**Applicability**

- **Cell banks**: MCB and each WCB
- **Cell types**: PCC, DCL, SCL, CCL

B.11.3.2.2 **Spiroplasma and others**

Other mollicutes such as spiroplasma may be introduced into cell substrates through contamination of raw materials (peptons) or due to the nature and permissivity of the cells (e.g. insect cells). According to the cell bank manufacturing process, if the raw material exposure is at the level of MCB or before, it may be appropriate to test the MCB only. If further exposure is possible, testing of the WCB may also be necessary.

Detection of such mollicutes may require adapted culture conditions (medium and/or temperature), depending on the strain to be detected. To guarantee a broad detection of the mollicutes, it is helpful to use NAT after suitable validation with an appropriate model (e.g. *Spiroplasma citri* or other strain according to the cell origin).

**Applicability**

- **Cell banks**: MCB, WCB (recommended for insect cells)
- **Cell types**: DCL, SCL, CCL (recommended for insect cell substrates and when raw materials of plant origin are used during the cell bank preparation or production process)

B.11.3.3 **Mycobacteria**

The test for mycobacteria is performed as described below or by a method approved by the NRA/NCL.
Inoculate 0.2 ml of the sample in triplicate on to each of two suitable solid media (such as Löwenstein–Jensen medium and Middlebrook 7H10 medium). Inoculate 0.5 ml in triplicate into a suitable liquid medium at 37 °C for 56 days.

In some countries, the incubation period is 42 days.

An appropriate positive-control test should be conducted simultaneously with the sample under evaluation, and the test should be shown to be capable of detecting the growth of small amounts of mycobacteria. In addition, the fertility of the medium in the presence of the preparation to be examined should be established by a spiking inoculation of a suitable strain of a *Mycobacterium* sp., such as bacille Calmette–Guérin (BCG). If at the end of the incubation time, no growth of mycobacteria occurs in any of the test media, and the positive control and spiked control show appropriate growth, the preparation complies with the test.

NAT may be used as an alternative to this culture method, provided that such an assay is shown to be comparable to the compendial culture method. An appropriate comparability study should be carried out that includes a comparison of the respective detection limits of the alternative method and culture method. Specificity (mycobacteria panel detected, potential false-positive results due to cross-reaction to other bacteria) should also be considered. An in vivo method, as described in the test in guinea-pigs, may also be used (see section B.11.2.1.3).

**Applicability**

- **Cell banks:** MCB or WCB
- **Cell types:** PCC, DCL, SCL, CCL

**B.11.4 Transmissible spongiform encephalopathies**

TSEs are a group of slowly developing fatal neurological diseases affecting the brains of animals and humans. The accepted view at present is that they are caused by non-conventional infectious agents known as prions (PrP\textsuperscript{tse}), which are made up of a normal host protein (PrP) in an abnormal conformation. TSEs include BSE of cattle, scrapie of sheep, CJD and its variant form (vCJD), GSS and FFI in humans, CWD in elk and deer, and transmissible mink encephalopathy (118, 119). Normal PrP (PrPc) protein may be expressed on cell surfaces, but in vivo this protein can misfold and become the abnormal disease-causing type PrP\textsuperscript{tse}, which is able to catalyse the conversion of PrPc protein into the abnormal conformation. Compared with PrPc, PrP\textsuperscript{tse} is relatively resistant to common proteolytic enzymes such as proteinase K.

BSE was first described in the United Kingdom in 1984, and the numbers of clinical cases there reached a peak in 1992–1993. Other countries were also affected. Currently, the number of new infections detected annually is low (120).
However, BSE remains a particular concern because cases still occur, albeit at a low rate, and there is a legacy arising from the prolonged incubation period of the disease, the life expectancy of cell banks, and the complexity of the processes by which they are established.

BSE in cattle has been transmitted to humans in the form of vCJD. Approximately 200 individuals have been affected either directly through exposure to BSE-infected material or through secondary transmission by non-leukocyte-depleted red blood cells. Classical CJD has also been transmitted by medical procedures, including administration of cadaveric growth hormone (121), corneal transplant and the use of dura mater, and vCJD may be transmissible by the same routes. vCJD has also been transmitted by human blood products. Although there is no evidence of vCJD transmission by plasma products, public health precautions have been implemented to minimize the possible risk of onward vCJD transmission by this route (122). Cattle-derived proteins, including serum, have often been used in the growth of cells in culture and the production of biological products, including vaccines and recombinant products. Thus, it is important to ensure that any ruminant-derived material used in biopharmaceutical manufacture is free of the agents that cause TSE. Moreover, as there is a possible but unquantifiable risk that cells can become infected by the agents of TSE, it is important that possibly contaminated ruminant material should be excluded from the start of the development of any cell line used. When there is insufficient traceability in the legacy of a cell line, a risk assessment should be undertaken to aid decision-making about the suitability of the cell line for the intended use. There is currently no practical validated test that can be used for biological products or cell line testing for the agents of TSE other than infection of susceptible species, where the experiments are very difficult because of the length of the incubation. More usable tests such as protein misfolding cyclic amplification (PMCA), which is analogous to PCR for nucleic acids, and epitope protection assays (123) are under investigation, but their performance characteristics when used to detect TSE agents in biological products or cell lines have not been defined. Strategies for minimizing risk have therefore focused so far on sourcing materials from countries believed to be at very low risk of infection and on substituting animal-derived materials with non-animal-derived materials.

B.11.4.1 Infectivity categories of tissues

Ruminant tissues are categorized by WHO and other scientific bodies such as the EMA into three categories (category A: high infectivity; category B: lower infectivity; category C: no detectable infectivity) (57, 124). Category A includes brain and category C includes materials such as testes and bile. Assays of improved sensitivity have shown infectivity in tissues such as muscle that were
previously thought to be free of infectious agents, and the implication is that, while certain tissues contain large amounts of infectivity, many other tissues may contain low levels that are difficult to detect (57, 124–126).

B.11.4.2 Control measures, sourcing and traceability

Where effective alternatives to ruminant-derived material are available, they should be used in cell culture and manufacturing procedures. Examples include: cell culture medium free of animal material; polysorbate and magnesium stearate of plant origin; enzymes, such as rennet, of microbial origin (used in lactose production); and recombinant insulin and synthetic amino acids. It should be noted that recombinant materials may themselves be exposed to animal materials, so this potential should be considered when choosing recombinant materials as alternatives. However, it is not always possible to use ingredients that are free of animal materials, and raw materials of non-ruminant origin. For example, fetal bovine serum may have to be used in the development of cell lines or for fermentation. Under these circumstances, the raw materials should be sourced from countries classified by the World Organisation for Animal Health (OIE) as negligible BSE risk (geographical BSE-risk level I, or GBR I), as classified by the European Food Safety Authority. Raw materials of category C may be sourced from countries that are classified as controlled risk, provided there is assurance that no cross-contamination with materials of category A or B could have occurred during collection and processing (with the caveat that, while they have undetectable levels of infectivity, it could conceivably be present). Manufacturers should maintain records so that the finished product from any batch is traceable to the origin of any ruminant ingredient used in its manufacture that may pose a risk of exposure to a TSE agent, and each ruminant ingredient is traceable to the finished product. This includes ingredients used to develop and produce the MCB and WCB and, as far as is possible, traceability should be to the derivation of the cell line itself. This traceability in both directions is important for appropriate regulatory action if new scientific research indicates that there is a risk of TSE infectivity in the materials used, or if the use of the products is associated with vCJD. Category A and B ruminant materials originating from BSE-enzootic countries should not be used in the production of biologicals under any circumstances. Because new BSE cases continue to occur despite feed bans, because suitable tests for TSE agents in raw materials are not available, and because developments in scientific research indicate the presence of pathological prions in materials of category C, the best approach to TSE safety is not to use animal-derived protein. The next best approach is to source raw materials from countries classified as free of BSE, bearing in mind that cases may be detected in future.
B.11.4.3 Tests

No suitable screening tests are currently available for TSE agents in raw materials of human/ruminant origin similar to serological or PCR assays for screening for viral agents. Newer tests are being developed to screen for the presence of TSE agents in blood (such as PMCA, epitope-protection assay and others). Such tests, once validated, could eventually become suitable for the screening of raw materials and cell banks.

Approximately 15% of human TSEs are associated with inherited mutations in the PrP gene. These familial, but transmissible, TSEs are associated with around 30 known pathogenic mutations or with insertions and deletions in the octapeptide-repeat region of PrP (127). The PrP gene of new human cell substrates should be sequenced to exclude the presence of these genetic changes.

B.12 Summary of tests for the evaluation and characterization of animal cell substrates

This section provides an overview of tests that are recommended for the evaluation and characterization of animal cell substrates proposed for use in the production of biological products. Not all of the tests are appropriate for all animal cell substrates, but each of them should be considered and a determination made as to its applicability for a given cell substrate in the context of its use to manufacture a specific product. In addition, the point(s) at which a test should be applied needs to be rationalized. The overall testing strategy should provide assurance that risks have been mitigated to reasonable levels for the product and for its intended use. The testing strategy should be agreed with the NRA/NCL.

B.12.1 Cell seed

The cell seed is generally derived from a cell or tissue source of interest because of its potential utility in the development of a biological product. In some cases, the cell may be expected to serve as the substrate for the production of multiple products. RCBs (see also section A.5.3) would be considered cell seeds. The cell seed is usually of limited quantity, so extensive testing is not feasible. Some of the seed is therefore used to produce a supply of cells in a quantity that allows more extensive testing, as well as providing a long-term source of cells for use in manufacturing. This secondary cell source is usually called the MCB. However, the cell seed also may be used to produce additional low-passage material that can be banked (pre-MCB) and used to generate MCBs that then are characterized as described in this document.

Tests on the cell seed can be carried out at any point before the establishment of the MCB. Usually, such tests are limited to obtaining information that is essential for making the decision to commit resources to the preparation of an MCB. Such tests typically include viability, morphology, identity (e.g.
karyotype, isoenzymes) and sterility (e.g. bacterial, fungal, mycoplasma). These data serve as important background information, but they cannot substitute for the full characterization of the MCB.

B.12.2 Master cell bank and working cell bank

In general the MCB will be developed to generate a sufficient quantity of cells to supply enough vials of cells to produce many WCBs over an extended period (usually years). MCBs typically contain at least 200 vials and often 1000 or more. There should also be a sufficient number of vials in the WCB to provide material for the characterization of the cell line.

Some tests on the WCB are conducted on cells recovered directly from the bank itself; other tests are conducted on cells that have been propagated to a passage at or beyond the level that will be used for production. In addition, some tests may be appropriate to use as in-process control tests. In such cases, they should be identified and described in the recommendations applicable to specific products.

Authors

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Dr P. Christian, National Institute for Biological Standards and Control, Potters Bar, England; Dr M. Deschamps, GlaxoSmithKline Biologicals, Rixensart, Belgium; Dr R.M. Dhere, Serum Institute of India, Pune, India; Dr C. Hutchens, Pfizer, St. Louis, MO, USA; Dr J. Lebron, Merck Research Laboratories, West Point, PA, USA; Dr I. Knezevic, World Health Organization, Geneva, Switzerland; Dr S. Lambert, World Health Organization, Geneva, Switzerland; Dr A. Lewis, Center for Biologics Evaluation and Research, Bethesda, MD, USA; Dr L. Mallet, Sanofi Pasteur, Marcy L’Etoile, France; Dr P. Nandapalan, Therapeutic Goods Administration, Woden, ACT, Australia; Dr K. Peden, Center for Biologics Evaluation and Research, Bethesda, MD, USA; Dr J. Petricciani, Consultant, Palm Springs, CA, USA; Dr R. Sheets, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA; Dr J. Shin, World Health Organization, Geneva, Switzerland; Dr Y. Sohn, Korea Food and Drug Administration, Seoul, Republic of Korea; Dr G. Stacey, National Institute for Biological Standards and Control, Potters Bar, England; Mrs C.A.M. van der Velden, Consultant, Groenekan, Netherlands; Dr R. Wagner, Paul-Ehrlich-Institute, Langen, Germany; Dr O. Wimalaratne, Medical Research Institute, Colombo, Sri Lanka; and Dr D.J. Wood, World Health Organization, Geneva, Switzerland.
Since then, several draft recommendations were prepared by the drafting group consisting of: Dr J. Petricciani, Consultant, Palm Springs, CA, USA; Dr R. Sheets, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA; Dr G. Stacey, National Institute for Biological Standards and Control, Potters Bar, England; and Dr I. Knezevic, World Health Organization, Geneva, Switzerland. These recommendations were reviewed by the WHO Study Group on Cell Substrates in 2008.

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Dr K.S. Ahn, Korea Food and Drug Administration, Seoul, Republic of Korea; Dr J.H. Blusch, Novartis, Basel, Switzerland; Dr da Silva Guedes Jr., Bio-Manguinhos/Fiocruz, Rio de Janeiro, Brazil; Dr M. Deschamps, GlaxoSmithKline Biologicals, Wavre, Belgium; Dr G. Dong, National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China; Dr B. Gauvin, Amgen Inc., Thousand Oaks, CA, USA; Dr H. Kavermann, Roche Diagnostics GmbH, Penzberg, Germany; Dr K. King, United States Food and Drug Administration, Bethesda, MD, USA; Dr I. Knezevic, World Health Organization, Geneva, Switzerland; Dr A. Lewis, Center for Biologics Evaluation and Research, Bethesda, MD, USA; Dr L. Mallet, Sanofi Pasteur, Marcy L’Etoile, France; Dr P. Minor, National Institute for Biological Standards and Control, Potters Bar, England; Dr P. Nandapalan, Therapeutic Goods Administration, Woden, ACT, Australia; Dr D. Onions, Invitrogen Corporation, Carlsbad, CA, USA; Dr K. Peden, Center for Biologics Evaluation and Research, Bethesda, MD, USA; Dr J. Petricciani, Consultant, Palm Springs, CA, USA; Ms E. Ika Prawahju, National Agency of Drug and Food Control, Jakarta, Indonesia; Dr R. Sheets, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA; Dr G. Stacey, National Institute for Biological Standards and Control, Potters Bar, England; Dr R. Wagner, Paul-Ehrlich-Institute, Langen, Germany; and Dr D.J. Wood, World Health Organization, Geneva, Switzerland.

On the basis of the comments received from a broad range of regulators, manufacturers of vaccines and other biologicals and other relevant experts in 2009, the draft recommendations were updated by the drafting group and posted on the WHO biologicals web site for public consultation from 4 to 31 May 2010.

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Dr C. Conrad, World Health Organization, Geneva, Switzerland; Dr H. Kang, World Health Organization, Geneva, Switzerland; Dr I. Knezevic, World Health Organization, Geneva, Switzerland; Dr J. Petricciani, Consultant, Palm
Springs, CA, USA; Dr R. Sheets, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA; Dr P. Minor, National Institute for Biological Standards and Control, Potters Bar, England; Dr D. Onions, Invitrogen Corporation, Carlsbad, CA, USA; Dr K. Peden, Center for Biologics Evaluation and Research, Bethesda, MD, USA; Dr J. Shin, World Health Organization, Geneva, Switzerland; and Dr Glyn Stacey, National Institute for Biological Standards and Control, Potters Bar, England.

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References


Appendix 1

Tests for bovine viruses in serum used to produce cell banks

Serum should be tested for adventitious agents such as bacteria, fungi, mycoplasmas and viruses, prior to use in the production of MCBs and WCBs. In addition, consideration should be given to risk-mitigation strategies, such as inactivation by heat or irradiation, to ensure that adventitious agents that were not detected in the manufacture and quality control of the serum will be inactivated to a degree acceptable to the NRA/NCL. If irradiation or other inactivation (e.g. heat sterilization) methods are used in the manufacture of the serum, the tests for adventitious agents should be performed prior to inactivation, to enhance the opportunity for detecting the contamination. If evidence of viral contamination is found in any of the tests, the serum is acceptable only if the virus is identified and shown to be present in an amount that has been shown in a validation study to be effectively inactivated. For serum that is not to be subjected to a virus inactivation/removal procedure, if evidence of viral contamination is found in any tests, generally the serum will not be acceptable. If the manufacturer chooses to use serum that has not been inactivated, thorough testing of the serum for adventitious agents, using current best practices, should be undertaken. If any viruses are identified in the serum, the cell banks made in this manner should be shown to be free of the identified virus(es).

If irradiation is used, it is important to ensure that a reproducible dose is delivered to all batches and to the component units of each batch. The irradiation dose must be low enough for the biological properties of the reagents to be retained, while being high enough to reduce virological risk. Therefore, irradiation delivered at such a dose may not be a sterilizing dose.

Factors to be considered in testing serum

Bovine serum can be contaminated by a wide range of viruses. Manufacturers typically produce very large pools of serum involving samples from up to a thousand animals. Consequently, many serum batches contain detectable genomic sequences of viruses such as BVDV and bovine polyomavirus (1), although this may represent contamination of the pool by one viraemic animal.

Other viruses are sporadic contaminants and may be regionally restricted, such as Cache Valley virus, BTV and epizootic haemorrhagic disease virus. In
some cases, contamination has been reported only on a few occasions, as in the case of calicivirus 2117 (2).

Application of new methods such as MPS has revealed new viruses, like the parvoviruses, some of which are frequent and high-level contaminants of serum (3, 4). The importance and potential pathogenicity of these viruses requires further investigation.

An important factor in infectivity assays is that virions might be neutralized by antibody in the serum pool. It is advisable to set limits for the level of BVDV-neutralizing antibody in serum pools, as this may mask the presence of potentially infectious virus.

There should be awareness of the statistical limits of screening assays in detecting viruses in large serum pools. For example, in an infection of a fermenter by Cache Valley virus, it was estimated that fewer than 10 viruses per litre were present in the serum and, at this low level, the virus escaped detection by conventional screening methods (5).

**General screening assay for infectious viruses**

A general screening assay typically involves culturing indicator cells over 21 days with test serum at 15% in the medium. At least two subpassages of the cells should be undertaken, usually at days 7 and 14. Detection of virus infection involves regular examination for the development of a CPE, haemadsorption assays and immunofluorescence (or other appropriate immunological detection method) for specific viruses. Immunofluorescence is particularly important for the detection of BVDV, as many isolates are non-cytopathic. At the end of the assay, cytological staining (e.g. with Giemsa stain) is used to reveal viral inclusions and other CPEs that were not detected during the direct observation of the live cells.

Indicator cells should be selected that are permissive for a wide range of bovine viruses. MDCK cells or bovine turbinate cells are often used, and it is also of value to include additional cells such as Vero cells.

The assay should be capable of detecting the following: BVDV, BPIV3, BPV1, rabies virus, RNO3, IBR, BRSV, BTV, bovine adenovirus 5 (BAV5), and vesicular stomatitis virus. Separate positive-control bottles of indicator cells should be infected with each of the viruses above, except rabies virus. In the case of rabies virus, slides of fixed infected cells should be used as a positive control for the immunofluorescent assay. Uninfected negative-control cells should also be established.

A typical assay involves the use of 75 cm² bottles containing the indicator cells and a total of ~250 ml of test serum, allowing for serum used during refeeding of the cells after passage.
Procedure
Assay set-up
Initially, negative-control bottles and test article bottles are established. The test article bottles are inoculated and maintained with the test serum at 15% in the medium. The negative-control bottles are mock infected with serum known to be free of detectable viruses. Passage of the cells is usually required on day 7.

Cells for the positive control are prepared from the negative control bottles on day 13 or 14 or when the cells are ≥70% confluent. The cells are subcultured into 25 cm² flasks (for immunofluorescence) and six-well plates (for haemadsorption and cytological staining).

The following day, the remaining negative-control and test article cells are subcultured to 75 cm² flasks for immunofluorescence and to six-well plates for haemadsorption and cytological staining.

Infection with positive controls
Coincident with the final subculture of test article and negative-control cultures, flasks of bovine turbinate cells are inoculated with the immunofluorescence positive control viruses BVDV, BAV5, BPV, BTV, BRSV, IBR and BPIV3. Plates of bovine turbinate cells are inoculated with BPIV3, the positive control for haemadsorption, and with cytopathic BVDV, the positive control for cytological staining. Likewise, Vero flasks are inoculated with REO3, the immunofluorescence positive control, and plates are inoculated with BPIV3, the haemadsorption and cytological staining positive control. All immunofluorescence positive-control viruses should be inoculated at 100–300 TCID₅₀ (median tissue culture infective dose).

Analysis
After a minimum of 21 days after inoculation, and at least 7 days after the last subculture (but earlier if CPE is observed), negative-control and test article cultures are assayed for haemadsorption and fixed for immunofluorescence and cytological staining. Cells from the positive-control flasks are transferred to multiwell slides and fixed for immunofluorescence when CPE involving ≥10% of the monolayer is observed, and stored at ≤−60 °C. Cells in the positive-control six-well plates are assayed for haemadsorption and cytological staining 7 days after inoculation, or when CPE is apparent. Haemadsorption involves testing at least one six-well plate with chicken and guinea-pig erythrocytes at 2–8 °C and at 20–25 °C.

Nucleic acid amplification assays for viruses
Nucleic acid amplification technologies such as PCR have utility in screening serum for sporadic contaminants and for those viruses where infectivity assays are not available. Nucleic acid extractions should be from a significant volume
(e.g. 25–50 ml) and the statistical limits for detection in the serum pool should be calculated. The presence of genomic sequences does not necessarily indicate the presence of infectious virus, although encapsidated genomes can be identified by treatment of the sample with nucleases prior to amplification. Some virus-inactivating or removal processes can be evaluated using NAT, by determining whether intact, full-length, amplifiable genomes are present before and after treatment.

**Specific in vitro infectivity assays**

Bovine polyomavirus is an important contaminant because it is able to infect primate cells (6), belongs to an oncogenic family of viruses and expresses a T-antigen that can transform primary cells into tumour cells (7). Furthermore, there is serological evidence of zoonotic infection (8). Infectious virus is not easily detected in conventional assays; a long period of culture and a NAT end-point or immunological end-point such as immunofluorescence should be used.

Other viruses are not easily detected in standard infectivity assays. For instance, calicivirus 2117 appears to be more permissive for replication in CHO cells than standard bovine cell lines used in in vitro infectivity assays. Similarly, while general screening methods will detect certain bovine adenoviruses, herpesviruses and parvoviruses, not all bovine viruses belonging to these families are detected.

**References**

Appendix 2

Tumorigenicity protocol using athymic nude mice to assess mammalian cells

During the characterization of an MCB (or WCB), the cells should be examined for tumorigenicity in a test approved by the NRA or the NCL.

The following model protocol is provided to assist manufacturers and NRAs/NCLs to standardize the tumorigenicity testing procedure so that the interpretation and comparability of data between various laboratories and regulatory authorities can be facilitated.

1. Test animals
The test article cell line and the control cells are each injected into separate groups of 10 athymic mice (Nu/Nu genotype) 4–7 weeks old.

Because male athymic mice often display aggressive traits against each other when housed together, loss of some mice during the observation period often occurs. Therefore, the use of only female mice should be considered.

2. Test article cells
Cells from the MCB or WCB that have been propagated to at least three population doublings beyond the limit for production are examined for tumorigenicity.

3. Control cells

a. Positive control cells
HeLa cells from the WHO cell bank are recommended as the positive control reference preparation. Portions of that bank are stored at the American Type Culture Collection (USA) and the National Institute for Biological Standards and Control (England).

Other cells may be acceptable to the NRA/NCL if HeLa cells from the WHO cell bank are not available.

b. Negative-control cells
Negative-control cells are not required. Databases (both published data and the unpublished records/data of the animal production facility that supplied the test animals) of rates of spontaneous neoplastic diseases in nude mice may be taken into account during the assessment of the results of a tumorigenicity test.
If negative-control cells are included, clear justification must be provided. In particular, the number of animals used must provide meaningful data, and the rationale for generating additional data must be persuasive to the NRA/NCL in the context of animal welfare regulations.

4. Validity
In a valid test, progressively growing tumours should be produced in at least 9 out of 10 animals injected with the positive control reference cells. At least 90% of the inoculated control and cells and test cells must be viable for the test to be valid.

5. Inoculum
The inoculum for each animal is \(10^7\) viable cells (except as described in 11.b, below), suspended in a volume of 0.1 ml PBS.

Cell culture medium without serum has been used in the past to suspend the cell inoculum. However, many current media are serum free and contain one or more growth factors that may affect the result of the tumorigenicity assay. Therefore, careful consideration should be given to the choice of the liquid into which the cells are suspended.

6. Injection route and site
The injection of cells may be by either the intramuscular or the subcutaneous route. If the intramuscular route is selected, the cells should be injected into the thigh of one leg. If the subcutaneous route is selected, the cells should be injected into the supraclavicular region of the trunk.

On the basis of findings of published studies, the intracerebral route may be more appropriate in some cases. For example, lymphoblastoid cells have been shown to proliferate best when inoculated by the intracerebral route.

7. Observation period
All animals are examined weekly by observation and palpation, for a minimum of 16 weeks (i.e. 4 months) for evidence of nodule formation at the site of injection when the route of inoculation is intramuscular or subcutaneous. Examinations need not be more frequent than two times a week for the first 3–6 weeks, and once a week thereafter.

In some countries, the observation period is 4–7 months, depending on the level of concern associated with the specific cell substrate in the context of the product being developed. Whether a longer observation period is needed should be agreed with the NRA/NCL. Also see 8 below.
8. Assessment of the inoculation site over time

If a nodule appears, it is measured in two perpendicular dimensions, the measurements being recorded weekly to determine whether the nodule grows progressively, remains stable or decreases in size over time. Animals bearing nodules that appear to be regressing should not be killed until the end of the observation period. Cell lines that produce nodules that fail to grow progressively are not considered to be tumorigenic.

If a nodule fails to grow progressively but persists during the observation period and retains the histopathological morphology of a neoplasm, this should be discussed with the NRA/NCL, to determine whether additional testing will be required. Such testing could include extending the observation period or switching to a newborn nude mouse, ATS-treated newborn rat, or other in vivo model, to assess the tumorigenicity of the cell substrate.

If the cells that are injected fail to form tumours or to persist during the 4-month observation period, it may be necessary to extend the observation period or switch to a newborn nude mouse, ATS-treated newborn rat, or other in vivo model to assess the tumorigenicity of the cell substrate. This will depend on the level of concern associated with the specific cell substrate in the context of the product being developed. Whether such additional testing is needed should be agreed with the NRA/NCL.

9. Final assessment of the inoculation site and other sites

At the end of the observation period, or at an earlier time if required due to the death of an animal or other justifiable circumstances, all animals (including the reference group(s)) are killed and examined for gross and microscopic evidence of the proliferation of inoculated cells at the site of injection and at other sites such as the heart, lungs, liver, spleen, kidneys, brain and regional lymph nodes, since some CCLs may give rise to tumours at distant sites without evidence of tumour at the injection site. The tissues are fixed in 10% formol saline and sections are stained with heamatoxylin and eosin for histological examination to determine whether there is evidence of tumour formation and metastases by the inoculated cells.

10. Assessment of metastases (if any)

Any metastatic lesions are examined further to establish their relationship to the primary tumour. If what appears to be a metastasis to a distant site differs histopathologically from the primary tumour, consideration should be given to the possibility that the tumour either developed spontaneously or was induced by one or more of the components of the cell substrate, such as an oncogenic virus.
If the histopathology or genotype of any tumours that develop are inconsistent with the inoculated cell type, or are of a histopathological type that has not been recognized as occurring spontaneously in the test species, additional tests should be undertaken to determine whether such tumours are actually spontaneous or are induced by elements within the cell substrate itself, such as oncogenic viruses or oncogenic DNA sequences. In such cases, appropriate follow-up studies should be discussed and agreed with the NRA/NCL.

11. Interpretation of results

a. The test in nude mice is considered positive if at least 2 out of 10 animals inoculated with the test article cells develop tumours that meet the following two criteria:
   
   i. Tumours appear at the site of inoculation or at a metastatic site.
   
   ii. Histological or genotypic examination reveals that the nature of the cells constituting the tumours is consistent with that of the inoculated cells.

   In the past, chromosomal markers have been useful to demonstrate that the tumour cells are of the same species as that from which the inoculated cells were derived. However, the use of cytogenetics for this purpose has largely been replaced by genetic and antigenic markers.

b. If only 1 out of 10 animals develops a tumour that meets the two criteria in 11.a, the cell line should be considered to be possibly tumorigenic and should be examined further. Such testing could include one or more of the following: repeating the test in an additional 10 nude mice, extending the observation period, increasing the size of the inoculum, or switching to the newborn nude mouse model, the ATS-treated newborn rat model, or other in vivo model. In such cases, appropriate follow-up investigations should be discussed and agreed with the NRA/NCL. For example, it may be appropriate to determine whether the tumour is of nude mouse origin and whether there are any viral or inoculated cell DNA sequences present.

Assessment of dose–response may provide additional information on the characteristics of the CCL. If such studies are undertaken, the design should be based on the in vivo titration of the inoculum in groups of 10 animals per dose level. For example, if 10 out of 10 animals develop tumours with an inoculum of $10^7$ cells, the titration could be done with $10^5$, $10^3$ and $10^1$ cells in groups of 10 animals each.
Appendix 3

Oncogenicity protocol for the evaluation of cellular DNA and cell lysates

When appropriate, and particularly for vaccines, cell DNA and cell lysates from tumorigenic cell substrates should be examined for oncogenicity in a test approved by the NRA/NCL.

In some countries, the following testing strategy is used:

1. Type of test animals

Newborn (i.e. <3 days old) nude mice, newborn hamsters and newborn rats have been used to assess the oncogenic potential of cell lines. At this stage, it is not possible to draw definitive conclusions on the relative sensitivity of the three animal assays for oncogenicity, and testing is recommended in each of them. When data on the ability of these models to detect oncogenic activity are obtained, this recommendation may change.

2. The point in cells’ life history at which they should be tested

Cells from the MCB or WCB, propagated to the proposed in vitro cell age for production or beyond, should be examined for oncogenicity. Three extra population doublings ensure that the results of the oncogenicity test can be used in the assessment of overall safety of the product, even under the assumption of a worst-case situation, and therefore provide a safety buffer.

3. Use of controls

The purpose of the positive control is to assure that an individual test is valid, by demonstrating that the animal model has the capacity to develop tumours from inoculated cell components (i.e. a negative result is unlikely to be due to a problem with the in vivo test). While an appropriate positive control for cell lysate oncogenicity assay is not clear, the recent description of an oncogene-expression plasmid for activated H-ras and c-myc has been shown to induce tumours in animals (1). As the test with cell lysates is designed primarily to detect oncogenic viruses rather than oncogenes, the use of DNA as a positive control may not be suitable, both because of the nature of the assay and because DNA may not be stable in a cellular lysate.

Whether a negative-control arm, such as PBS, is included should be discussed with the NRA/NCL. An advantage of including a negative-control arm
is that the frequency of tumour induction with lysates is expected to be low and may approximate to the spontaneous tumour frequency in the indicator rodent, providing an important comparison to the test article arm.

4. Number of test animals
While the number of animals in a tumorigenicity test can be 10 per group, the number in an oncogenicity test should be larger, owing to the lower expected tumour incidence. The number per group should be discussed with the NRA/NCL.

5. Inoculation of test material
a. Cell lysate
A lysate of the cells should be prepared by a method that avoids virus disruption, while allowing maximum virus release and ensuring that all cells are lysed (e.g. three freeze/thaw cycles, followed by low-speed centrifugation). Each animal should be inoculated subcutaneously above the scapula with a lysate obtained from $10^7$ cells. Before inoculation, it should be determined that no viable cells are present, as development of tumours from cells would invalidate the test. The cell lysate is suspended in PBS and inoculated in a volume of 50–100 µl into newborn nude mice, newborn hamsters and newborn rats. If, at the end of the observation period, there is no evidence of a progressively growing tumour at the site of inoculation or at distant sites, the cell line may be considered not to possess oncogenic activity. If tumours are observed in this assay, the species of origin will need to be confirmed. The species of tumours that arises in a tumorigenicity assay will be that of the cell substrate, while the species of tumours that arises in an oncogenicity assay is that of the host (e.g. rodent). If the cells were not lysed properly, it may be that the tumours that arose were from the species of the cell substrate.

b. DNA
Total cellular DNA isolated from the cell substrate should be inoculated subcutaneously above the scapulae in PBS into newborn nude mice, newborn hamsters and newborn rats. The amount of DNA inoculated should be ≥100 µg in 50–100 µl. Because of the concentrations necessary to achieve ≥100 µg of DNA, it may be necessary to shear the DNA; this can be done by sonication or by several passes in a needle and syringe. A positive-control plasmid with the test article DNA should be inoculated into a few mice, to confirm that the cellular DNA is not inhibitory and that the animals are susceptible to tumour induction by DNA.
6. Observation period
Animals are examined weekly by observation and palpation, for evidence of nodule formation at the site of injection. The observation period should last at least 4 months.

7. Assessment of the inoculation site over time (progressive or regressive growth)
If one or more nodules appear, they are measured in two perpendicular dimensions, the measurements being recorded weekly to determine whether the nodule grows progressively, remains stable or decreases in size over time. Animals bearing nodules that are progressing should be killed when the nodule reaches a size of approximately 2 cm in diameter, unless a lower limit has been established by the authorities for the humane treatment of animals.

8. Final assessment of the inoculation site
At the end of the observation period, all animals, including the reference group(s), are killed and examined for gross and microscopic evidence of tumour formation at the site of injection and at other sites. Any tumour that is identified is divided into three equal parts: (a) fixed in formalin for histopathology; (b) used to establish a cell line, when possible; and (c) frozen for subsequent molecular analysis.

9. Evaluation of animals for metastases
Animals are examined for microscopic evidence of metastatic lesions in sites such as the liver, heart, lungs, spleen and regional lymph nodes.

10. Assessment of metastases (if any)
All tumours are examined to establish their relationship to the primary tumour at the site of inoculation. If what appears to be a metastatic tumour differs histopathologically from the primary tumour, it is necessary to consider the possibility that this tumour developed spontaneously. This may require further testing of the tumour itself. In such cases, appropriate follow-up studies should be discussed and agreed with the NRA/NCL.

11. Interpretation of results
If tumours arise in the cell lysate or DNA assay, these could be induced by an oncogenic virus or oncogenic DNA. Because of the implications for the use of a cell substrate that contains an oncogenic agent or an oncogenic activity for a biological, the NRA/NCL should be consulted to consider additional
experiments to identify the oncogenic agent/activity and to determine the suitability of the use of the CCL.

**Applicability**

- *Cell banks:* MCB or WCB taken beyond EOPC level/ECB
- *Cell types:* CCL, SCL (recommended when tumorigenic cells are used in vaccine production)

**References**

# Annex 4

## Recommendations to assure the quality, safety and efficacy of recombinant hepatitis B vaccines


### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Introduction</td>
<td>191</td>
</tr>
<tr>
<td>2. General considerations</td>
<td>192</td>
</tr>
<tr>
<td>Part A. Manufacturing recommendations</td>
<td>193</td>
</tr>
<tr>
<td>A.1 Definitions</td>
<td>193</td>
</tr>
<tr>
<td>A.2 General manufacturing recommendations</td>
<td>195</td>
</tr>
<tr>
<td>A.3 Control of source materials</td>
<td>195</td>
</tr>
<tr>
<td>A.4 Fermentation</td>
<td>197</td>
</tr>
<tr>
<td>A.5 Single harvests</td>
<td>198</td>
</tr>
<tr>
<td>A.6 Control of aqueous bulk (purified antigen bulk)</td>
<td>199</td>
</tr>
<tr>
<td>A.7 Final vaccine bulk</td>
<td>203</td>
</tr>
<tr>
<td>A.8 Filling and containers</td>
<td>204</td>
</tr>
<tr>
<td>A.9 Control tests on the final vaccine lot</td>
<td>204</td>
</tr>
<tr>
<td>A.10 Records</td>
<td>207</td>
</tr>
<tr>
<td>A.11 Retained samples</td>
<td>207</td>
</tr>
<tr>
<td>A.12 Labelling</td>
<td>207</td>
</tr>
<tr>
<td>A.13 Distribution and shipping</td>
<td>207</td>
</tr>
<tr>
<td>A.14 Stability testing, storage and expiry date</td>
<td>207</td>
</tr>
<tr>
<td>Part B. Nonclinical evaluation</td>
<td>208</td>
</tr>
<tr>
<td>B.1 Strategy for cloning and expressing the gene product</td>
<td>208</td>
</tr>
<tr>
<td>B.2 Characterization of purified HBsAg for new vaccines</td>
<td>209</td>
</tr>
<tr>
<td>B.3 Animal models</td>
<td>210</td>
</tr>
<tr>
<td>B.4 Nonclinical safety studies</td>
<td>210</td>
</tr>
<tr>
<td>B.5 Toxicology studies</td>
<td>210</td>
</tr>
<tr>
<td>Part C. Clinical evaluation</td>
<td>211</td>
</tr>
<tr>
<td>C.1 Consideration for clinical studies</td>
<td>211</td>
</tr>
<tr>
<td>C.2 Assessment of the immune response</td>
<td>212</td>
</tr>
<tr>
<td>C.3 Clinical studies</td>
<td>212</td>
</tr>
<tr>
<td>C.4 Post-marketing studies</td>
<td>215</td>
</tr>
<tr>
<td>Part D. Recommendations for national regulatory authorities</td>
<td>216</td>
</tr>
<tr>
<td>D.1 General</td>
<td>216</td>
</tr>
<tr>
<td>D.2 Release and certification</td>
<td>216</td>
</tr>
</tbody>
</table>
Recommendations published by WHO are intended to be scientific and advisory. Each of the following sections constitutes guidance for national regulatory authorities (NRAs) and for manufacturers of biological products. If an NRA so desires, these recommendations may be adopted as definitive national requirements, or modifications may be justified and made by the NRA. It is recommended that modifications to these recommendations be made only on condition that the modifications ensure that the vaccine is at least as safe and efficacious as that prepared in accordance with the recommendations set out below. The parts of each section printed in small type are comments for additional guidance intended for manufacturers and NRAs, which may benefit from these details.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-HBs</td>
<td>antibody to HBsAg</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>ED$_{50}$</td>
<td>median effective dose</td>
</tr>
<tr>
<td>GMT</td>
<td>geometric mean titre</td>
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<tr>
<td>ICH</td>
<td>International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use</td>
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<tr>
<td>IU</td>
<td>International Unit</td>
</tr>
<tr>
<td>HBsAg</td>
<td>hepatitis B surface antigen</td>
</tr>
<tr>
<td>MCB</td>
<td>master cell bank</td>
</tr>
<tr>
<td>MPL</td>
<td>monophosphoryl lipid A</td>
</tr>
<tr>
<td>NAT</td>
<td>nucleic acid amplification test</td>
</tr>
<tr>
<td>NIBSC</td>
<td>National Institute for Biological Standards and Control</td>
</tr>
<tr>
<td>NRA</td>
<td>national regulatory authority</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>WCB</td>
<td>working cell bank</td>
</tr>
</tbody>
</table>

## 1. Introduction

These Recommendations are intended to provide national regulatory authorities (NRAs) and vaccine manufacturers with background and guidance on the production, quality control and evaluation of the safety and efficacy of recombinant hepatitis B vaccines for prophylactic use.

The first document outlining the Requirements for the production and control of hepatitis B vaccines containing hepatitis B surface antigen (HBsAg) purified from the plasma of chronically infected individuals was adopted by the Expert Committee on Biological Standardization in 1980 (1) and later revised in 1987 (2).

Following the development of hepatitis B vaccines containing HBsAg produced by recombinant DNA techniques in yeast, a new set of Requirements...
was developed subsequent to a meeting of experts in 1985 (3) and was adopted by the Committee in 1986 (4). These Requirements were revised to include vaccines produced by recombinant techniques in mammalian cells as well as yeast cells, in 1988 (5).

With the development and implementation of new in vitro assays to determine antigen content, an amendment was published to include the use of the in vitro assay in the quality control of recombinant hepatitis B vaccines (6).

The current document applies to vaccines containing HBsAg only and will replace the WHO Requirements for hepatitis B vaccine made by recombinant DNA techniques, published as Annex 2 in WHO Technical Report Series, No. 786 (5) and with a corresponding amendment in Annex 4 of WHO Technical Report Series, No. 889 (6). It should be read in conjunction with all other relevant WHO guidelines, including those on nonclinical and clinical evaluation of vaccines (7, 8).

2. General considerations

Hepatitis B virus has several characteristics that distinguish it from the other families of DNA viruses. It has an outer coat (more substantial than a membrane or envelope) consisting of protein, lipid and carbohydrate, and bearing a unique antigen complex, HBsAg. Its nucleic acid consists of a circular DNA genome of relative molecular mass of about 2 million, part of which is double stranded and part single stranded, which is an unusual feature among viruses. Virus recovered from the plasma of a hepatitis B carrier was used to clone the HBsAg gene.

The HBsAg gene has been inserted into yeast and mammalian cells by means of appropriate expression vectors. Antigen expressed in several species of yeast – namely Saccharomyces cerevisiae, Pichia pastoris and Hansenula polymorpha – and Chinese hamster ovary (CHO) cells has been used to produce hepatitis B vaccines for more than 20 years. Electron microscopy revealed that purified HBsAg obtained from transfected cultures exists as particles that are 15–30 nm in diameter, with the morphological characteristics of free surface antigen in plasma. Purified antigen has been shown to induce antibodies in mice and guinea-pigs and to protect chimpanzees from infection with hepatitis B virus.

All hepatitis B vaccines currently on the market require formulation with adjuvants. Preservatives are used for multidose presentations but there are some single-dose presentations available without preservative. Recombinant hepatitis B vaccines are available as monovalent products or included in combination vaccines together with other antigens such as hepatitis A virus, diphtheria toxoid, tetanus toxoid, whole-cell or acellular pertussis components, Haemophilus influenzae type b conjugated antigen and inactivated poliomyelitis viruses.

The Recommendations that follow apply to the manufacture, quality control, and nonclinical and clinical evaluation of hepatitis B vaccines containing
HBsAg made by recombinant DNA methods. It is expected that new or significantly modified recombinant hepatitis B vaccine formulations will be characterized according to the recommendations made in Part A and Part B of this document and assessed in clinical studies, as described in Part C.

Particular emphasis is placed on the introduction of “in-process” controls to monitor consistency of production, in addition to the tests on the final product. Certain tests will be required on every batch of vaccine, whereas others will be required only to support licensure or significant manufacturing changes.

The vaccine lots used in clinical trials should be adequately representative of the formulation and manufacturing scale intended for marketing.

**Part A. Manufacturing recommendations**

**A.1 Definitions**

**A.1.1 International name and proper name**

The international name should be “recombinant hepatitis B vaccine”. The proper name should be the equivalent of the international name in the language of the country of origin. The use of the international name should be limited to vaccines that satisfy the requirements described below.

**A.1.2 Descriptive definition**

The recombinant hepatitis B vaccine is a preparation of purified HBsAg that has been produced by recombinant DNA techniques. The antigen may be formulated with a suitable adjuvant.

**A.1.3 International reference materials**

International standards and reference reagents for the control of potency of hepatitis B vaccine are not available. Therefore, product-specific reference preparations may be used.

The Second International Standard for HBsAg (non-adjuvanted HBsAg) subtype adw2, genotype A contains 33 International Units (IU) per vial. This material is intended as a quantitative reference standard for HBsAg subtype adw2, genotype A, and the use of this standard will give an indication of the analytical sensitivity of an assay for the detection of HBsAg. The International Standard for HBsAg should not be used as a vaccine reference.

An International Standard for hepatitis B immunoglobulin is available for use in assays designed to quantify antibody to HBsAg (anti-HBs) in human serum. Antibody responses to hepatitis B vaccines should be expressed in IU. The Second
International Standard for hepatitis B immunoglobulin (2008) was prepared from fractionated human plasma and freeze-dried in ampoules. It has an assigned potency of 100 IU/ampoule. This preparation is in the custody of the National Institute for Biological Standards and Control (NIBSC), Potters Bar, England.

A.1.4 Terminology

Adjuvant: a vaccine adjuvant is a component that potentiates the vaccine's immune response to an antigen and/or modulates it towards the desired immune responses.

Adventitious agents: contaminating microorganisms of the cell substrate or source materials used in its culture that may include bacteria, fungi, mycoplasmas and endogenous and exogenous viruses that have been unintentionally introduced.

Aqueous bulk: purified antigen bulk before the addition of an adjuvant.

Anti-HBs: Antibodies to HBsAg (see below).

Cell bank: a collection of containers (e.g. ampoules, vials) containing aliquots of a suspension of cells from a single pool of cells of uniform composition, stored frozen under defined conditions (typically <−60 °C for yeast, and in liquid nitrogen for mammalian cell lines).

End-of-production cells: a cell suspension containing the cells harvested at the end of culture/fermentation.

Final vaccine bulk: the formulated bulk, prepared from one or more batches of aqueous bulk (purified antigen) to which adjuvant has been added, present in the container from which the final containers are filled.

Final vaccine lot: a collection of sealed final containers of vaccine that is homogeneous with respect to the risk of contamination during the filling process. A final vaccine lot must therefore have been filled from a single vessel of final bulk in one working session.

Hepatitis B virus: a 42-nm double-shelled virus particle, originally known as the Dane particle, which contains the DNA genome of the virus.

HBsAg: hepatitis B surface antigen, comprising a complex of antigens associated with the virus envelope and subviral forms (22 nm spherical and tubular particles). Native HBsAg is encoded by envelope gene sequences (S plus pre-S) in the viral DNA. Recombinant DNA-derived hepatitis B vaccines may contain the S gene product or products of the S/pre-S combination.

Master cell bank (MCB): a collection of containers containing aliquots of a suspension of cells from a single pool of cells of uniform composition, stored frozen under defined conditions (typically <−60 °C for yeast, and in liquid nitrogen for mammalian cell lines). The MCB is used to derive all working cell banks (WCBs) for the anticipated lifetime of the vaccine production.

Production cell culture: a cell culture derived from one or more containers of the WCB used for the production of vaccines.
Single harvest: the biological material prepared from a single production run.

Working cell bank (WCB): a collection of containers containing aliquots of a suspension of cells from a single pool of cells of uniform composition, derived from the MCB, stored frozen under defined conditions (typically $<-60^\circ\text{C}$ for yeast, and in liquid nitrogen for mammalian cell lines). One or more aliquots of the WCB are used for routine production of the vaccine. Multiple WCBs are made and used during the lifetime of the vaccine production.

A.2 General manufacturing recommendations

The general manufacturing requirements contained in WHO’s Good manufacturing practices for pharmaceutical products (9) and Good manufacturing practices for biological products (10) should apply to the establishment of facilities for manufacturing hepatitis B vaccine, with the addition of the following:

- production areas should be decontaminated before they are used for the manufacture of hepatitis B vaccine;
- hepatitis B vaccine should be produced by staff who have not handled animals or infectious microorganisms on the same working day. The staff should be persons who have been examined medically and have been found to be healthy;
- no cultures of microorganisms or eukaryotic cells, other than those approved by the NRA, should be introduced into or handled in the production area at any time during manufacture of the vaccine.

A.3 Control of source materials

A.3.1 Cell substrates for antigen production

The use of any cell substrate should be based on a cell bank system. The NRA should be responsible for approving the cell bank. Only cells that have been approved by and registered with the NRA should be used to produce HBsAg protein. Appropriate history of the cell bank should be provided.

A.3.1.1 Yeast cells

The characteristics of the recombinant production strain (i.e. host cell in combination with the expression vector system) should be fully described, and information should be given on the absence of adventitious agents and on gene homogeneity for the MCBs and WCBs. A full description of the biological characteristics of the host cell and expression vectors should be given. The physiological measures used to promote and control the expression of the cloned gene in the host cell should be described in detail. This should include the genetic
markers of the host cell, the construction, genetics and structure of the expression vector, and the origin and identification of the gene that is being cloned.

The nucleotide sequence of the gene insert, and of adjacent segments of the vector and restriction-enzyme mapping of the vector containing the gene insert, should be provided as required by the NRA. Characterization of the gene product (HBsAg) should be provided in support of licensure (see Part B).

MCBs and WCBs should be tested for the absence of adventitious bacteria and fungi, according to Part A of the General requirements for the sterility of biological substances (11) or by a method approved by the NRA. Cells must be maintained in a frozen state that allows recovery of viable cells without alteration of genotype. The cells should be recovered from the frozen state, if necessary, in selective media such that the genotype and phenotype consistent with the unmodified host and unmodified recombinant DNA vector are maintained and are clearly identifiable. Cell banks should be identified and fully characterized by means of appropriate tests.

Where appropriate, plasmid retention in the cell bank should be monitored at regular intervals. Data that demonstrate the stability of the expression system during storage of the recombinant WCB up to or beyond the passage level used for production should be provided and approved by the NRA. Any instability of the expression system occurring in the seed culture, or after a production-scale run (end-of-production cells), should be documented.

### A.3.1.2 Mammalian cells

If mammalian cells are used, the cell substrate and cell banks should conform to WHO’s Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (12). Other relevant guidelines provide additional information (13). Cell substrates and cell banks should be approved by the NRA.

The maximum population doublings (or number of passages) allowable between the MCB, the WCB and the production cells should be approved by the NRA. The MCB is produced in sufficient quantities and stored in a secure environment, and is used as the source material to make manufacturers’ WCB. In normal practice, an MCB is expanded by serial subculture up to a population doubling (or passage number, as appropriate) selected by the manufacturer and approved by the NRA, at which point the cells are combined in a single pool, distributed into containers (e.g. ampoules, vials) and preserved cryogenically to form the WCB.

Tests are performed on the MCBs and WCBs in accordance with WHO’s Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (12) and Guidelines for assuring the quality of pharmaceutical and biological products prepared by recombinant DNA technology (14) and should be approved by the NRA.
A.3.2  **Cell culture medium**

If serum is used for the propagation of mammalian cells, it should be tested to demonstrate freedom from bacteria, fungi and mycoplasmas, according to WHO requirements (11). Suitable tests for detecting viruses in bovine serum are given in the Recommendations for the evaluation of animal cell cultures as substrates for manufacture of biological medicinal products and for the characterization of cell banks (12).

Validated molecular tests for bovine viruses may replace the cell culture tests of bovine sera. As an additional monitor of quality, sera may be examined for freedom from phage and endotoxin. Gamma irradiation may be used to inactivate potential contaminant viruses.

The acceptability of the source(s) of any components of bovine, porcine, sheep or goat origin should be approved by the NRA. These components should comply with the current version of the *WHO Guidelines on animal transmissible spongiform encephalopathies in relation to biological and pharmaceutical products* (15). If trypsin is used for preparing cell cultures, it should be tested and found free of bacteria, fungi, mycoplasmas and infectious viruses – especially bovine or porcine paroviruses – as appropriate. The methods used to ensure this should be approved by the NRA. The trypsin should be gamma irradiated.

Human serum should not be used. However, human serum albumin may be used if it complies with WHO's Requirements for the collection, processing and quality control of blood, blood components and plasma derivatives (16). In addition, human albumin and materials of animal origin should comply with current *WHO Guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products* (15).

Penicillin and other beta-lactams should not be used at any stage of manufacture, as they are highly sensitizing substances. Other antibiotics may be used in the manufacture, provided that the quantity present in the final product is acceptable to the NRA.

Non-toxic pH indicators may be added (e.g. phenol red at a concentration of 0.002%). Only substances that have been approved by the NRA may be added.

A.4  **Fermentation**

A.4.1  **Production of cell cultures**

Only cell cultures derived from the WCB should be used for production. All processing of cells should be carried out in an area where no cells or organisms are handled other than those directly required for the process. The medium used should comply with the requirements given in section A.3.2.

A.4.1.1  **Control of HBsAg production up to single harvest in yeast expression system**

Microbial purity in each fermentation vessel should be monitored at the end of the production run, by methods approved by the NRA. Any agent added to the
fermenter or bioreactor in order to feed cells or to induce/increase cell density should be approved by the NRA.

A.4.1.2 Control of HBsAg production up to single harvest in mammalian cells

Production of cell cultures should be carried out under conditions agreed with the NRA. These conditions should include details of the culture system used, the cell doubling time, the number of subcultures or the duration of the period of subcultivation permitted, and the incubation temperature. Cell culture vessels should be monitored for potential microbial contamination both during and at the end of the production runs, by methods approved by the NRA.

A.5 Single harvests

A.5.1 Storage and intermediate hold times

During the purification process, all intermediates should be maintained under conditions shown by the manufacturer as retaining the desired biological activity. Hold times should be approved by the NRA.

A.5.2 Tests on single harvest

A.5.2.1 Sampling

Samples required for the testing of single harvests should be taken immediately on harvesting, prior to further processing. For mammalian cell cultures, if the tests for adventitious agents are not performed immediately, the samples taken for these tests should be kept at a temperature of –60 °C or below and subjected to no more than one freeze/thaw cycle.

A.5.2.2 Test for bacteria, fungi and mycoplasma contamination

Bacterial and fungal contamination in the cell culture vessels should be monitored during and at the end of the production runs, by methods approved by the NRA. If mammalian cells are used in production, each single harvest or pool of single harvests should be shown by appropriate tests to be free from bacteria, fungi and mycoplasma contamination (11).

Nucleic acid amplification test (NAT) techniques alone or in combination with cell culture, together with an appropriate detection method, may be used as an alternative to one or both of the compendial mycoplasma-detection methods after suitable validation and agreement by the NRA, as described in WHO’s Recommendations for the evaluation of animal cell cultures as substrates for manufacture of biological medicinal products and for the characterization of cell banks (12).
A.5.3 **Consistency of yield**
Data should be provided on the consistency of yield between runs and during individual production runs, and the NRA should approve the criteria for an acceptable production run.

A.5.4 **Plasmid retention**
A sample of cells that are representative of each harvest must be tested to confirm that the recombinant phenotype has been retained. The method used should be approved by the NRA.

When the production method has been shown consistently to yield harvests that comply with the requirement for plasmid retention, the test may be omitted on the harvest after approval by the NRA. However, the stability of the vector should be monitored regularly on the WCB. Particular attention should be paid to the plasmid copy number during conditions of storage and recovery.

Where the plasmid is integrated in the host cell genome, the presence of the integrated HBsAg gene insert should be confirmed.

A.5.5 **Tests for adventitious agents if mammalian cells are used in production**
Each single harvest or pool of single harvests should be tested for adventitious agents in cell cultures in accordance with the Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (12).

Additional testing may be performed using NATs.

A.6 **Control of aqueous bulk (purified antigen bulk)**
The purification procedure can be applied to a single harvest, to a part of a single harvest, or to a pool of single harvests. The maximum number of single harvests that may be pooled should be approved by the NRA. Adequate purification may require several purification steps, based on different principles. This will minimize the possibility of co-purification of extraneous cellular materials. The methods used for the purification of the HBsAg should be appropriately validated and approved by the NRA. Any agent added during the purification process should be documented and its removal adequately validated and tested for, as appropriate (see section A.6.1.8).

The monovalent purified antigen bulk may be stored under conditions shown by the manufacturer to retain the desired biological activity. Intermediate hold times should be approved by the NRA. Additional tests on intermediates during the purification process may be used to monitor the consistency/yields.
A.6.1 Tests on the aqueous bulk (purified antigen)

The aqueous bulk should be tested according to the tests outlined below. All quality control release tests and specifications for aqueous bulk should be validated and approved by the NRA.

A.6.1.1 Purity

The degree of purity of each aqueous bulk should be assessed by suitable methods. Examples of suitable methods of analysing the proportion of potential contaminating proteins in the total protein of the preparation are polyacrylamide gel electrophoresis (PAGE), optionally followed by densitometric analysis, or high-performance liquid chromatography. Other methods include automated electrophoresis systems. The aqueous bulk must be not less than 95% pure.

A.6.1.2 Protein content

The protein content should be determined by using the micro-Kjeldahl method, the Lowry technique or another suitable method.

A.6.1.3 HBsAg content

The HBsAg content of the aqueous bulk should be determined by an appropriate immunochemical method. An appropriate reference material should be included in these assays, so that consistency of the production is monitored. This reference material could be a representative bulk of known HBsAg and protein content, or a highly purified preparation of HBsAg of known HBsAg and protein content, with an acceptable stability profile; it should be stored in single-use aliquots. It is important to note that reference materials based on adjuvanted product are not suitable for use in assays of non-adjuvanted intermediate bulks of HBsAg.

The ratio of HBsAg content to protein content should be determined. The antigen/protein ratio should be within the limits approved by the NRA.

A.6.1.4 Identity

The test for antigen content will generally serve as confirmation of the identity of the protein in the bulk. Alternatively, immunoblots using HBsAg-specific antibodies in the assessment of purity could also serve to confirm the molecular identity of the product. Such tests should be approved by the NRA.

A.6.1.5 Lipids

The lipid content of each aqueous bulk should be determined by an appropriate method. Both the methods used and the permitted concentrations of lipid should be approved by the NRA. This test may be omitted for routine lot release, upon
demonstration of consistency of the purification process to the satisfaction of the NRA.

A.6.1.6 Carbohydrates
The carbohydrate content of each aqueous bulk should be determined by an appropriate method. Both the methods used and the permitted concentrations of carbohydrates should be approved by the NRA. This test may be omitted for routine lot release, upon demonstration of consistency of the purification process to the satisfaction of the NRA.

A.6.1.7 Sterility tests for bacteria and fungi
Each aqueous bulk should be tested for freedom from bacteria and fungi in accordance with WHO requirements (11), or by a method approved by the NRA.

A.6.1.8 Tests for agents used during purification or other phases of manufacture
The aqueous bulk should be tested for the presence of any potentially hazardous agents used during manufacture. The method used and the concentration limits should be approved by the NRA. This test may be omitted for routine lot release, upon demonstration that the purification process consistently eliminates the agent from the purified bulks.

Where a monoclonal antibody is used in vaccine preparation (e.g. for immunological affinity chromatography to purify HBsAg), the antibody used should be characterized and its purity determined. The product should be tested for residual antibody. The methods used and the permitted concentrations of antibody should be approved by the NRA.

Several NRAs have drafted guidelines for the control of monoclonal antibody preparations used for the manufacture of biological products for human use.

If the HBsAg has been treated with formaldehyde and/or other agents, the material should be tested for the presence of free formaldehyde and/or the other agents. The method used and the permitted concentration should be approved by the NRA.

A.6.1.9 Tests for residuals derived from the antigen expression system
The amount of residuals derived from the antigen-expression system (e.g. DNA or host-cell proteins) should be determined in each purified bulk of monovalent antigen, by sensitive methods. In the case of yeast-derived products, these tests may be omitted for routine lot release, upon demonstration that the purification process consistently eliminates the residual components from the monovalent bulks to the satisfaction of the NRA.
For mammalian cells, acceptable limits of residual cellular DNA for specific products should in general be set in consultation with the NRA/NCL. The characteristics of the cell substrate, the intended use of the vaccine and, most importantly the effect of the manufacturing process on the size, quantity and biological activity of the residual cellular DNA fragments, should be considered as outlined in WHO's Recommendations for the evaluation of animal cell cultures as substrates for manufacture of biological medicinal products and for the characterization of cell banks (12).

One licensed vaccine produced in mammalian cells contains less than 100 pg DNA per dose.

For products produced in yeast, residual cellular DNA is considered an impurity.

One licensed vaccine produced in yeasts contains less than 10 pg cellular DNA per dose.

A.6.1.10 Bacterial endotoxins

Each final aqueous bulk should be tested for bacterial endotoxins. The endotoxin content should be consistent with levels found to be acceptable in vaccine lots used in clinical trials; the limits should be approved by the NRA.

A.6.1.11 Albumin content (when mammalian cells are used)

If animal serum is used in mammalian cell cultures, or at any stage in the manufacturing process, testing should be carried out to assess the residual serum in the purified bulk. The concentration of animal serum in the vaccine should be not more than 50 ng per human dose of vaccine.

A.6.2 Adjuvant bulk

A.6.2.1 Tests on adjuvants

Hepatitis B vaccines may contain the immunostimulant monophosphoryl lipid A (MPL) adsorbed onto aluminium compounds (e.g. aluminium phosphate). When the adsorption of the MPL is performed prior to the final formulation step, the degree of adsorption of MPL should be determined using a suitable method (e.g. gas chromatography). The test for completeness of adsorption of the MPL may be omitted, upon demonstration of process consistency and/or if performed on the final vaccine lot.

A.6.2.2 Sterility tests

Each adjuvant bulk should be tested for bacterial and fungal sterility according to WHO requirements (11).
A.6.2.3  pH  
The pH of each adjuvant bulk should be tested and shown to be within the range of values approved by the NRA.

A.7  Final vaccine bulk  
The final vaccine bulk may consist of one or more purified aqueous bulks. Only aqueous bulks that have satisfied the requirements outlined in previous sections should be formulated into the final vaccine bulk. The antigen concentration in the final formulation should be sufficient to ensure a dose that is consistent with that shown to be safe and effective in human clinical trials. Formulation is generally based on protein content, but HBsAg content may be used.

It should be noted that formulation based on HBsAg may be affected by changes in the kits and/or reagents used to determine antigen content. This should be considered and included in validation/bridging studies when kit changes occur (see Appendix 1).

The operations necessary for preparing the final vaccine bulk should be conducted in a manner that avoids contamination of the product. In preparing the final vaccine bulk, any substances such as diluents, stabilizers or adjuvants that are added to the product should have been shown, to the satisfaction of the NRA, not to impair the safety and efficacy of the vaccine in the concentration used. Until the bulk is filled into containers, the final vaccine bulk suspension should be stored under conditions shown by the manufacturer to retain the desired biological activity.

A.7.1  Tests on the final vaccine bulk  
All tests and specifications for the final vaccine bulk, unless otherwise specified, should be approved by the NRA.

The HBsAg may be formulated with other vaccine antigens into a combined vaccine (e.g. HAV, DTwP-HBsAg, DTaP-HBsAg). Specific issues related to the formulation and quality control of final vaccine bulk of combination vaccines will be addressed in a separate document (17).

A.7.1.1  Sterility tests  
Each final vaccine bulk should be tested for bacterial and fungal sterility, according to WHO requirements (11).

A.7.1.2  Adjuvants  
Each final vaccine bulk should be assayed for the content of adjuvants. This test may be omitted if it is performed on the final vaccine lot. Where aluminium
compounds are used, the content of aluminium should not be greater than 1.25 mg per single human dose.

If an immunostimulant (e.g. monophosphoryl lipid A) is present, each final vaccine lot should be assayed for the immunostimulant content. This test may be omitted if it is performed on the final vaccine bulk.

A.7.1.3 Degree of adsorption
The degree of adsorption of HBsAg antigen present in the final vaccine bulk should be assessed. This test may be omitted upon demonstration of the process consistency to the satisfaction of the NRA, or if the test is performed on the final vaccine lot.

A.7.1.4 Preservative content
The final vaccine bulk should be assayed for preservative content, if added. This test may be omitted if it is performed on the final vaccine lot.

A.7.1.5 Potency
If an in vivo potency test (i.e. immunogenicity) is used, this test may be performed on the final vaccine bulk. The methods for detecting antibodies to HBsAg and for analysing data should be approved by the NRA. The vaccine potency should be compared with that of a reference preparation and the NRA should determine the limits of potency and should approve the reference preparation used. If an in vitro potency test is performed, it should be performed on every lot of final vaccine. Methodological considerations regarding potency assays are outlined in Appendix 1.

A.8 Filling and containers
The requirements concerning filling and containers given in WHO's Good manufacturing practices for biological products (10) should apply to vaccine filled in the final form.

Care should be taken to ensure that the materials of which the container and, if applicable, transference devices and closure are made do not adversely affect the quality of the vaccine. The manufacturers should provide the NRA with adequate data to prove the stability of the product under appropriate conditions of storage and shipping.

A.9 Control tests on the final vaccine lot
Samples should be taken from each final vaccine lot for testing and should fulfil the requirements of this section. All the tests and specifications, including the methods used and the permissible limits for the different parameters listed under this section, should, unless otherwise specified, be approved by the NRA.
A.9.1 **Inspection of containers**
Each container of each final vaccine lot should be inspected visually or mechanically, and containers showing abnormalities should be discarded.

A.9.2 **Appearance**
Visual inspection of the appearance of the vaccine should be described with respect to the physical form and colour.

A.9.3 **Identity**
The vaccine should be identified as HBsAg by appropriate methods. The assay used for potency may serve as the identity test. For manufacturers producing vaccines both with and without MPL, the final vaccine lot should also be identified by checking for the presence (or not) of MPL.

A.9.4 **Sterility tests**
Each final vaccine lot should be tested for bacterial and fungal sterility, according to WHO requirements (11), or by other acceptable methods.

A.9.5 **General safety (innocuity) test**
Each final lot should be tested for the absence of abnormal toxicity in mice and guinea-pigs, using a general safety (innocuity) test approved by the NRA, and should pass the test. This test may be omitted for routine lot release once consistency of production has been established to the satisfaction of the NRA.

A.9.6 **pH and osmolality**
The pH and osmolality of a pool of final containers should be tested. The test for osmolality may be omitted once consistency of production is demonstrated to the satisfaction of the NRA.

A.9.7 **Preservatives**
Each final vaccine lot should be tested for preservative content, if added. This test may be omitted if it is performed on the final vaccine bulk.

A.9.8 **Pyrogen/endotoxin content**
The vaccine in the final container should be tested for pyrogenic activity, either by intravenous injection into rabbits or by a *Limulus* amoebocyte lysate test. Endotoxin content or pyrogenic activity should be consistent with levels found to be acceptable in vaccine lots used in clinical trials and approved by the NRA.
The test is conducted until consistency of production is demonstrated to the satisfaction of the NRA.

A.9.9 **Assay for adjuvant**

Each final vaccine lot should be assayed for adjuvant content. Where aluminium compounds are used, the amount of aluminium should not be greater than 1.25 mg per human dose.

Should an immunostimulant (e.g. MPL) be present, each final vaccine lot should be assayed for the immunostimulant content. This test may be omitted if it is performed on the final vaccine bulk.

A.9.10 **Degree of adsorption**

The degree of adsorption of the antigen and, where applicable, of MPL to the aluminium compounds (aluminium hydroxide or hydrated aluminium phosphate) in each final vaccine lot should be assessed if this test is not performed on the MPL bulk or final bulk. This test may be omitted for routine lot release, upon demonstration of the product consistency to the satisfaction of the NRA.

A.9.11 **Potency tests**

An appropriate quantitative test for potency by an in vivo or in vitro method should be performed on samples that are representative of the final vaccine lot. The method and the analysis of data should be approved by the NRA. If an in vivo potency test is performed on the final bulk, the test on the final container may be omitted. Methodological considerations regarding potency assays are outlined in Appendix 1.

Because of the diversity in the reactivity of vaccines produced by different manufacturing techniques and differences in the adjuvants used for the formulation, it is unlikely that an International Standard will be suitable for the standardization of assays of vaccines from all manufacturers. Manufacturers should therefore establish a product-specific reference preparation that is traceable to a lot of vaccine shown, in clinical trials, to be efficacious. The NRA should approve the reference preparation used and agree with the potency limits applied. The performance of this reference vaccine should be monitored by trend analysis using relevant test parameters, and the reference vaccine should be replaced when necessary.

The stability of the product-specific reference vaccine may also be monitored by routine assay against a stable HBsAg preparation. The inclusion of such a preparation in assays at regular intervals would monitor the stability of the product-specific reference vaccine and would monitor test/kit performance. However, this preparation would not be intended for use in establishing potency values.
A.10 Records
The requirements of WHO's Good manufacturing practices for biological products (10) should apply.

A.11 Retained samples
The requirements of WHO's Good manufacturing practices for biological products (10) should apply.

A.12 Labelling
The requirements of WHO's Good manufacturing practices for biological products (10) should apply, with the addition of the information that follows.

The label on the carton, the container or the leaflet accompanying the container should state:

- the nature of the cells used to produce the antigen;
- the nature of any preservative and the amount of adjuvant present in the vaccine;
- the volume of one recommended human dose, the immunization schedules, and the recommended routes of administration (this information should be given for newborn babies, children, adults and immunosuppressed individuals, and should be the same for a given vaccine for all regions of the world);
- the amount of HBsAg protein contained in one recommended human dose.

A.13 Distribution and shipping
The requirements of WHO's Good manufacturing practices for biological products (10) should apply.

In addition, the conditions of shipping should be such as to ensure that the adjuvanted vaccine does not freeze. Temperature indicators should be packaged with each vaccine shipment to indicate whether freezing occurs. Further guidance is provided in the WHO model guidance for the storage and transport of time- and temperature-sensitive pharmaceutical products (18). If freezing has occurred, the vaccine should not be used.

A.14 Stability testing, storage and expiry date
A.14.1 Stability testing
Adequate stability studies are an essential part of vaccine development. Current guidance on the evaluation of vaccine stability is provided in the WHO Guidelines
for stability evaluation of vaccines (19). Stability testing should be performed at different stages of production – namely on single harvests, aqueous bulk, final vaccine bulk and final vaccine lot – to validate their claimed shelf-life. The stability of the vaccine in its final form, and at the recommended storage temperatures, should be demonstrated to the satisfaction of the NRA on final containers from at least three lots of final product. The formulation of HBsAg antigens and adjuvant must be stable throughout its shelf-life. Acceptable limits for stability should be agreed with national authorities.

A.14.2 Storage conditions
The final container vaccine should be kept at 2–8 °C. If other storage conditions are used, they should be fully validated by appropriate stability studies and approved by the NRA. The vaccine should have been shown to maintain its potency for a period equal to that between the date of release and the expiry date. During storage, liquid-adsorbed vaccines should not be frozen.

A.14.3 Expiry date
The expiry date should be fixed upon the approval of the NRA, and should take account of the experimental data on stability of the vaccine.

Some manufacturers base the expiry date on the date of formulation of the final bulk. Others base the expiry date on the date of the last satisfactory potency test (i.e. the date on which the animals were inoculated with the vaccine in an in vivo test, or the date of the in vitro potency test performed on the final container).

Part B. Nonclinical evaluation
Nonclinical evaluation of hepatitis B vaccines should be based on the WHO guidelines on nonclinical evaluation of vaccines (7). The following issues should be considered in the context of the development of new recombinant hepatitis B vaccines. Prior to clinical testing of any new or modified hepatitis B vaccine in humans, there should be extensive product characterization, immunogenicity testing, safety testing and proof-of-concept studies in animals.

B.1 Strategy for cloning and expressing the gene product
A full description should be given of the biological characteristics of the host cell and expression vectors used in production. This should include details of: (i) the construction, genetics and structure of the expression vector; (ii) the origin and identification of the gene that is being cloned; and (iii) potential retrovirus-like particles in, and genetic markers for, mammalian cell-based expression systems.
The physiological measures used to promote and control the expression of the cloned gene in the host cell should be described in detail.

Data should be provided to demonstrate the stability of the expression system during storage of the WCB and beyond the passage level used for production. Any instability of the expression system occurring in the seed culture or after a production-scale run – for instance, involving rearrangements, deletions or insertions of nucleotides – must be documented. The NRA should approve the system used.

B.2 **Characterization of purified HBsAg for new vaccines**

Rigorous identification and characterization of recombinant DNA-derived vaccines is required as part of the application for marketing authorization. The ways in which these products differ chemically, structurally, biologically or immunologically from the naturally occurring antigen must be fully documented. Such differences could arise during processing at the genetic or post-translational level, or during purification.

B.2.1 **Characterization of gene products**

The molecular size of the expressed protein and its composition should be established by techniques such as sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by silver staining under reducing and non-reducing conditions or N-terminal sequencing by the Edman degradation method.

The identity of the protein should be established by peptide mapping and/or terminal amino acid sequence analysis. Following SDS-PAGE, the protein bands should be identified in immunoblots, using specific antibodies (e.g. monoclonal antibodies) to confirm the presence of the expected products of the HBsAg gene. The primary structure of the protein should be further characterized by suitable methods such as partial amino-acid sequence analysis and by peptide mapping. Mass spectrometry may be used to confirm the average molecular mass and the presence of the protein in the preparation.

Since it is known that conformational epitopes are essential for efficacy, it is essential to determine the morphological characteristics of the HBsAg particles and degree of aggregation. In addition, the protein, lipid, nucleic acid and carbohydrate content should be characterized and measured. Particle characterization may be done by atomic force microscopy, transmission electron microscopy, or dynamic light scattering.

The gene products from lots produced during vaccine development should be shown to possess antigenic determinants characteristic of HBsAg, by means of tests with monoclonal antibodies or polyclonal antibodies of defined specificity, directed against epitopes of HBsAg known to be relevant to the protective efficacy of the vaccine.
B.3  Animal models
There is no adequate, relevant animal model for hepatitis B infection other than the chimpanzee. The efficacy of recombinant HBsAg vaccines has been demonstrated in challenge studies in this model by several manufacturers and, therefore, such studies are no longer required for new vaccines based on the HBsAg protein.

The immunogenicity of new HBsAg vaccines and existing vaccines for which there has been a significant manufacturing change should be evaluated in nonclinical studies (e.g. in rabbits, guinea-pigs, mice and possibly nonhuman primates). The nonclinical programme should take into account the following.

- The titres of anti-HBsAg should be directly compared between the candidate vaccine and at least one licensed comparator (preferably one for which there has been extensive clinical use and generation of data supporting its effectiveness in routine use). If testing is performed due to a significant change in manufacturing, then the candidate vaccine should be compared with the corresponding licensed vaccine.

- If it is proposed that a new candidate vaccine will contain an adjuvant, its inclusion should be supported by adequate immunogenicity data that, in addition to measuring humoral antibody, may include an assessment of the cellular immune response. Studies should compare the adjuvanted candidate vaccine with the HBsAg alone and/or with HBsAg administered in conjunction with a well-established adjuvant (such as an aluminium salt).

- There may be a need to evaluate other antibody responses and/or cellular immune responses, in order to characterize the immune response in more depth.

B.4  Nonclinical safety studies
As no effects other than on the immune system are expected with hepatitis B vaccines in the absence of specific toxins, safety pharmacological studies are not required.

B.5  Toxicology studies
Toxicology studies should be undertaken in accordance with the WHO guidelines on nonclinical evaluation of vaccines (7). Such studies should reflect the intended clinical use of the vaccine in babies and young children.

If the vaccine is formulated with a novel adjuvant, nonclinical toxicology studies should be conducted as appropriate for the adjuvant concerned. Repeated dose toxicity studies may be used to compare the safety profile of the novel
adjuvant with the safety profile of an established vaccine formulation, taking into account existing guidelines.

If a novel cell substrate (i.e. a substrate that has not been previously licensed or used in humans) is used for the production of a hepatitis B vaccine, safety aspects, such as potential immune responses elicited by residual host cell proteins, should be investigated in a suitable animal model.

Variations in the route of administration or in the vaccine formulation require evaluation of immunogenicity of the hepatitis B vaccine, together with adequate animal safety/toxicological studies, taking into account existing guidelines (7, 8).

Part C. Clinical evaluation

This section addresses the clinical evaluation of new hepatitis B vaccines and of existing vaccines for which it is proposed to make a significant change to the manufacturing process. The content and extent of the clinical programme will vary according to each possible scenario, and it is recommended that vaccine-specific requirements for clinical studies are discussed with the appropriate NRAs.

C.1 Consideration for clinical studies

In general, clinical trials should adhere to the principles described in the WHO guidelines for good clinical practice (GCP) for trials on pharmaceutical products (20). General principles described in the WHO guidelines on clinical evaluation of vaccines: regulatory expectations (8) apply to hepatitis B vaccines and should be followed. Some of the issues that are specific to the clinical development programme for hepatitis B vaccines are discussed in the following sections and should be read in conjunction with the general guidance mentioned above. These recommendations should be viewed in the light of further data on the safety and immunogenicity of hepatitis B vaccines and any relevant data on similar types of vaccines that may become available.

After more than 20 years of clinical use of recombinant HBsAg vaccines, in addition to the experience gained from use of the early plasma-derived vaccines, there is sufficient experience to support the approval of new candidate vaccines (including those that may contain a novel adjuvant) and major changes to the manufacturing of existing vaccines based on clinical studies that assess safety and immunogenicity in seronegative subjects.

Infant immunization is the most effective strategy for preventing hepatitis B infection and this approach has been incorporated in the immunization programmes of more than 177 countries (21). However, catch-up strategies, adult vaccination and the vaccination of special populations are common, and studies that address different types of usage are considered in the following sections.
C.2 **Assessment of the immune response**

The assessment of the immune response should be based on measurement of the anti-HBsAg antibody concentration in serum, using a validated and standardized assay. An International Standard for hepatitis B immunoglobulin (anti-HBs) has been established and should be used in the assays to determine antibody responses in immunogenicity clinical studies.

The use of validated quantitative assays is critical for the evaluation of immune responses. Testing should be conducted by laboratories that implement quality assurance of testing procedures. Assay validation data should be reviewed and approved by the NRA. Assay validation involves demonstration that the performance characteristics of the method meet the requirements for its intended use (22). The protocols for assay validation studies should identify and justify the choice of the parameters to be studied, along with the predefined acceptance criteria. The validation report should include a detailed description of the processing and storage of samples, reference standards and reagents, and generation of the calibration curve.

See section C3.1 regarding analysis of the immunogenicity data.

C.3 **Clinical studies**

New hepatitis B vaccines should be compared directly with at least one licensed vaccine for which there is considerable clinical experience in routine use. The selection of the comparator should be discussed with the NRA, and selection should take into account the total antigen content of the candidate vaccine and the study population. Where possible, it is preferable for a candidate vaccine (whether monovalent or not) to be compared with a monovalent licensed vaccine. However, this may not be feasible in studies in infants, owing to the need to deliver numerous antigens concomitantly using multivalent HBsAg-containing vaccines. More information regarding the clinical evaluation of combination vaccines containing hepatitis B is discussed in a separate document (17).

In studies performed to support major changes to the manufacture of a licensed vaccine, the candidate vaccine should be compared with the existing vaccine (i.e. manufactured according to the licensed process).

New HBsAg vaccines should usually be tested initially in healthy adult volunteers. It is important to take into account that the immune response to HBsAg is age-dependent and decreases with the increasing age of adults. After the age of 30–40 years, antibody levels indicative of protection are achieved after a primary vaccination series in less than 90% of subjects, and in only 65–75% of vaccinees aged over 60 years (21). Thus, studies may restrict enrolment of adults by age, or may employ age stratification.

Once immunogenicity is demonstrated in adults, further studies may be conducted in younger target populations according to the intended use.
(e.g. neonates, infants). As one of the most important uses of the hepatitis B vaccine is to prevent infection in infants born to carrier mothers, unless a true efficacy trial is performed in this group, clinical studies may potentially include a measure of the kinetics of antibody acquisition in comparison to vaccines that have an established efficacy in this situation, rather than a simple comparison of seropositive rates or geometric mean titres (GMTs) following the last dose of the primary series. As the birth dose is never in combination with other vaccines, the comparison could be made following the first dose, which is particularly important in the prevention of maternal transmission of hepatitis B virus.

The amount of recombinant HBsAg administered per dose requires justification on the basis of nonclinical studies and, if necessary, formal dose-ranging studies in adults. However, these may not always be necessary if the nonclinical data and mode of manufacturing are considered to support the dose that is appropriate at least for the initial clinical studies. In this regard, it should be noted that it is usual that lower doses of HBsAg are administered to subjects aged less than 12–15 years, compared to adults (i.e. half the adult dose is commonly used in infants and children up to a selected age). Therefore any new recombinant HBsAg-containing vaccine should be evaluated for dose-related immunogenicity according to age.

The primary series schedule(s) that are examined will probably follow those that are already approved for other recombinant HBsAg-containing vaccines according to specific target populations. However, if a candidate vaccine is proposed to contain an antigen dose and/or an adjuvant that is considerably different from that of licensed vaccines, then a formal evaluation of schedule may be necessary, according to specific populations (e.g. by age and/or other host factors).

Enrolment should usually be limited to subjects who have no history of hepatitis B vaccination or disease. It is preferred that studies should screen participants prior to enrolment for the presence of HBsAg or anti-HBc antibody. If the results of these tests are available only after the first vaccination is given, any subjects with a positive result should be eliminated from the primary analysis of immunogenicity.

Studies in neonates may include those born to HBsAg-positive and/or HBsAg-negative mothers, depending on the study objectives, and may stratify neonates accordingly. Studies in infants may be limited to those born to HBsAg-negative mothers (with no birth dose of hepatitis B-specific immunoglobulin), with or without a prior birth dose of vaccine according to the objectives, and may employ stratification accordingly.

One other scenario that requires a specific clinical development programme concerns vaccines that contain high doses of antigen and/or an adjuvant and that are intended for populations known to respond poorly or not
at all to standard primary courses. In such cases, the studies should be tailored according to the properties of the vaccine (in order to justify the dose of antigen and the adjuvant content in specific target populations). It may be appropriate to enrol subjects who have shown no detectable antibody response to a complete primary series, in order to evaluate the benefit of a higher dose and/or an adjuvanted vaccine (see also section C.3.3).

C.3.1 Immunogenicity end-points

C.3.1.1 Primary analysis

The protective efficacy of hepatitis B vaccines has been shown to be directly related to the induction of anti-HBs antibody. An anti-HBs antibody concentration of \( \geq 10 \text{ mIU} \) is generally considered to be a marker of protection against hepatitis B \(^{21}\) and studies should determine the percentage of seronegative persons who achieve this antibody level at approximately 4 weeks after completion of a primary series.

For the comparison between the candidate and reference vaccines, the protocol and analysis plan should predefine a well-justified non-inferiority margin \(^{23}\) to compare the percentages of subjects with \( \geq 10 \text{ mIU/ml} \) anti-HBs.

C.3.1.2 Secondary analysis

In all studies, it is appropriate that protocols should plan at least for a secondary analysis of percentages that achieve \( \geq 100 \text{ mIU/ml} \) anti-HBs and should present reverse cumulative distributions \(^{24}\). In addition, it is recommended that secondary analyses should compare the GMTs between vaccines, and studies may plan for a formal comparison of GMT ratios.

It is expected that at least some of the clinical studies, including those in the primary target population(s), should be conducted with different lots manufactured using the same process as that for the vaccine intended for the market. However, as indicated in WHO’s Guidelines on clinical evaluation of vaccines: regulatory expectations \(^{8}\), a formal clinical trial to demonstrate lot-to-lot consistency is not normally required unless there is a particular concern about the manufacturing consistency of the product and the potential impact that this may have on the efficacy and safety of the vaccine. If performed, lot-to-lot consistency should be designed in accordance with the principles outlined in section B.3.3.3 of WHO’s Guidelines on clinical evaluation of vaccines: regulatory expectations \(^{8}\).

C.3.2 Persistence of anti-HBs antibody

Long-term observations of efficacy in various age groups have indicated that, owing to the effect of persistence of immune memory, loss of detectable anti-HBs antibody in subjects who responded satisfactorily to a primary series does
not necessarily indicate lack of protection. Long-term follow-up studies from various epidemiological settings have confirmed that HBsAg-carrier status or clinical hepatitis B virus disease rarely occurs in subjects who responded to a primary series, even when the anti-HBsAg concentrations decline to ≤10 mIU/ml over time (21). However, the persistence of ≥10 mIU/ml anti-HBsAg should be evaluated for any new hepatitis B vaccine. The total duration of serological follow-up should be discussed and planned in advance with NRAs.

It is current opinion that additional doses of HBsAg-containing vaccine may not be needed after completion of the initial vaccination series (the occasional exception being routine use of an additional dose in toddlers who received a course with certain vaccines during infancy). Thus, it may be difficult to justify administration of a further dose solely to assess immune memory. However, it is of interest and potential benefit to administer an additional dose of an HBsAg-containing vaccine to subjects who have failed to maintain ≥10 mIU/ml anti-HBsAg. In these instances, the titres obtained after the additional dose should be compared with the titres observed shortly after the last dose of the initial vaccination series. Careful attention should be paid to the documentation of safety associated with additional doses.

C.3.3 Studies in special populations
There are several host factors that have been described in association with lack of response or poor response to hepatitis B vaccines (e.g. male sex, age over 40 years, smoking, obesity and several underlying diseases that include advanced HIV infection, chronic renal failure, chronic hepatic disease and diabetes). Clinical studies may be conducted to specifically assess the safety and immunogenicity of new recombinant HBsAg-containing vaccines in populations at risk of not responding adequately to vaccination. The design of such studies should take into consideration the potential need for a higher antigen dose and/or adjuvant.

C.3.4 Concomitant administration with other vaccines
The potential for immune interference between hepatitis B vaccines and other routine vaccines that may need to be given at the same time for convenience should be investigated, in order to make recommendations regarding concomitant use.

C.4 Post-marketing studies
The manufacturer has a responsibility to assess safety and effectiveness following initial approval of a new hepatitis B vaccine, particularly when formulated with other components as part of a combination vaccine. NRAs should ensure that adequate pharmacovigilance plans are in place regarding these activities at the time of first licensure. There should be specific commitments made by manufacturers to provide data to NRAs on a regular basis and in accordance
with national regulations. The data that are collected and submitted to the responsible NRAs should be assessed rapidly, so that action can be taken if there are implications for the marketing authorization.

The collection of reliable and comprehensive data on effectiveness involves close cooperation between manufacturers and public health authorities. Therefore, pre- and post-approval discussions between vaccine manufacturers responsible for placing the product on the market, and national and international public health bodies, are essential for ensuring that reliable effectiveness data are collected in the post-marketing period in selected countries/regions.

**Part D. Recommendations for national regulatory authorities**

**D.1 General**

The general recommendations for control laboratories given in the WHO guidelines for national authorities on quality assurance for biological products (25) should apply. These guidelines specify that no new biological substance should be released until consistency of manufacturing and quality, as demonstrated by a consistent release of batches, has been established. The detailed production and control procedures, and any significant changes in them, should be discussed with, and approved by, the NRA. For control purposes, the NRA should obtain the working reference from the manufacturers.

**D.2 Release and certification**

A vaccine lot should be released only if it fulfils the national requirements and/or Part A of the current Recommendations. A protocol based on the model is provided in Appendix 2; this should be signed by the responsible official of the manufacturing establishment, and should be prepared and submitted to the NRA in support of a request for release of the vaccine for use.

A statement signed by the appropriate official of the NRA should be provided if requested by a manufacturing establishment and should certify whether or not the lot of vaccine in question meets all national requirements, as well as Part A of these Recommendations. The certificate should also state the lot number, the number under which the lot was released, and the number appearing on the labels of the containers. In addition, the date of the last satisfactory HBsAg potency test, as well as the assigned expiry date on the basis of shelf-life, should be stated. A copy of the official national release document should be attached. The certificate should be based on the model given in Appendix 3. The purpose of the certificate is to facilitate the exchange of recombinant hepatitis B vaccines between countries.
Authors and acknowledgements

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April 2008 meeting
Temporary advisers: Dr M. Ferguson, National Institute of Biological Standards and Control, Potters Bar, England; Dr C. Conrad, Paul-Ehrlich-Institute, Langen, Germany; Dr D. Kim, Korea Food and Drug Administration, Chungcheongbuk-do, Republic of Korea; Ms D. Kusmiaty, National Quality Control Laboratory of Drug and Food, Jakarta, Indonesia; Dr M. Powell, Medicines and Healthcare Products Regulatory Agency, London, England; Dr C. Milne, European Directorate for the Quality of Medicines and HealthCare, Strasbourg, France; Dr A.L. Sterling, Centro para el Control Estatal de la Calidad de los Medicamentos, Havana, Cuba; Dr R. Gibert, Agence Française de Sécurité Sanitaire des Produits de Santé, Lyons, France; representatives from the Developing Country Vaccine Manufacturers’ Network (DCVMN): Mr. L. Jin, Beijing Tiantan Biological Product Co. Ltd, Beijing, China; Dr Y. Park, Berna Biotech Korea Corp., Incheon, Republic of Korea; Dr K. Suresh, Serum Institute of India Ltd, Pune, India; representatives from the International Federation of Pharmaceutical Manufacturers and Associations (IFPMA): Dr P. Bhuyan, Merck & Co., Inc., Upper Gwynedd, PA, United States of America (USA); Dr M. Duchène, GlaxoSmithKline Biologicals, Wavre, Belgium; Dr D. Felnerova, Berna Biotech Crucell Company, Berne, Switzerland; WHO Secretariat: Dr I. Knezevic; Dr N. Dellepiane; Dr C.R. Hernandez; Dr J. Joung.

August 2008 meeting
Temporary advisers: Ms E.I.P. Arisetianingsih, National Quality Control Laboratory of Drug and Food, Jakarta, Indonesia; Dr M. Baca-Estrada, Health Canada, Ottawa, Canada; Dr N.H. Cuong, National Institute of Quality Control for Vaccines and Biologicals, Nha Trang, Viet Nam; Dr R. Dobbelaer, WHO Adviser, Brussels, Belgium; Dr M. Ferguson, National Institute of Biological Standards and Control, Potters Bar, England; Mrs T. Jivapaisarnpong, Ministry of Public Health, Nonthaburi, Thailand; Mr D. Kim, Korea Food and Drug Administration, Chungcheongbuk-do, Republic of Korea; Dr S. Khomvilai, Queen Saovabha Memorial Institute, Bangkok, Thailand; Ms C. Kleyn, National Control Laboratory
for Biological Products, Bloemfontein, South Africa; Dr Z. Liang, National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China; Dr A. Maes, Scientific Institute of Public Health, Brussels, Belgium; Dr C. Milne, European Directorate for the Quality of Medicines, Council of Europe, Strasbourg, France; Dr S. Morgeaux, Agence Française de Sécurité Sanitaire des Produits de Santé, Lyons, France; Dr P. Natakul, Ministry of Public Health, Nonthaburi, Thailand; Dr S-R. Pakzad, Food and Drugs Control Laboratory, Ministry of Health and Medical Education, Tehran, Islamic Republic of Iran; Dr A.L. Sterling, Centro para el Control Estatal de la Calidad de los Medicamentos, Havana, Cuba; Professor N. Thu Van, National Institute of Hygiene and Epidemiology, Hanoi, Viet Nam; representatives from DCVMN: Ms I.S. Budiharto, Biofarma, Jakarta, Indonesia; Dr R.J. Chaganti, Shantha Biotechnics Ltd., Hyderabad, India; Mr K. Gopinathan, Bharat Biotech International Ltd, Hyderabad, India; Lic. M. Izquierdo Lopez, Centro de Ingenieria Genetica y Biotecnologia, Havana, Cuba; Dr J.J. Lee, LG Life Sciences, Seoul, Republic of Korea; Mr J.Y. Park, Berna Biotech Korea Corp., Incheon, Republic of Korea; Mr M.S.R. Sarma, Indian Immunologicals Ltd, Hyderabad, India; Dr J. Singh, Biological E. Ltd., Hyderabad, India; Mr A. Sood, Panacea Biotec Ltd., New Delhi, India; Dr K. Suresh, Serum Institute of India Ltd, Pune, India; Ms E. Wunsch, The Biovac Institute, Pinelands, South Africa; WHO Secretariat: Dr N. Dellepiane; Dr J. Joung; Dr A. Khadem; Mr D. Kumar; Ms C.A. Rodriguez.

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Temporary advisers: Dr C. Conrad, Paul-Ehrlich-Institute, Germany; Dr R. Dobbelaer, WHO Adviser, Belgium; Dr M. Ferguson, WHO Adviser, Norfolk, England; Dr R. Gibert, Agence Française de Sécurité Sanitaire des Produits de Santé, Lyons, France; Dr P. Hubrechts, Staten Serum Institut, Copenhagen, Denmark; Ms T. Jivapaisarnpong, Ministry of Public Health, Nonthaburi, Thailand; Dr D. Kusmiaty, National Quality Control Laboratory of Drug and Food, Jakarta, Indonesia; Dr A. Maes, Scientific Institute of Public Health, Brussels, Belgium; Dr B. Meade, WHO Adviser, USA; Dr S-R. Pakzad, Food and Drug Control Laboratory, Tehran, Islamic Republic of Iran; Dr H.C. Song, Korea Food and Drug Administration, Chungcheongbuk-do, Republic of Korea; Dr A.L. Sterling, Centro para el Control Estatal de la Calidad de los Medicamentos, Havana, Cuba; Dr D. Wilkinson, National Institute for Biological Standards and Control, Potters Bar, England; representatives from DCVMN: Dr P.V.V.S. Murthy, Biological E. Ltd, Hyderabad, India; Dr V.K. Srinivas, Bharat Biotech International Ltd, Hyderabad, India; Dr X. Yang, Wuhan Institute of
Biological Products, Wuhan, China; representatives from IFPMA: Dr J-M. Jacquet, GlaxoSmithKline Biologicals, Wavre, Belgium; Mr L. Nencioni, Crucell, Berne, Switzerland; Ms I. Pierard, GlaxoSmithKline Biologicals, Wavre, Belgium; Ms S. Uhlrich, Sanofi Pasteur, Lyons, France; WHO Secretariat: Dr M. Baca-Estrada; Dr N. Dellepiane; Dr I. Knezevic; Dr D. Lei; Dr S. Nishioka; Dr C. Rodriguez; Dr D.J. Wood.

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References


Appendix 1

Methodological considerations: potency tests for recombinant hepatitis B vaccines

Background
Recombinant hepatitis B vaccines were licensed in the mid-1980s. WHO published Requirements for hepatitis B vaccines produced by recombinant DNA techniques in yeast and mammalian cells in 1989 (1) and these were revised to include the use of in vitro potency tests in 1997 (2).

Because of the diversity in the reactivity of vaccines containing HBsAg produced by different manufacturing processes, and to which different adjuvants or immunostimulants have been added, recombinant hepatitis B vaccines produced by different manufacturers must be considered as different products. In view of these differences, it is unlikely to be possible to establish an International Standard that would be suitable for the standardization of assays of vaccines from all manufacturers. Furthermore, the stability of such a vaccine is unlikely to be adequate for long-term use and for the calibration of secondary standards. Manufacturers should therefore establish a product-specific reference preparation that is traceable to a lot of vaccine shown to be efficacious in clinical trials. This vaccine will serve as a working standard and will be included in all potency tests. The NRA approves the reference preparation used and the potency limits applied. The performance of this reference vaccine should be monitored by trend analysis using control charts of relevant test parameters (e.g. median effective dose \(ED_{50}\) for in vivo assays), and the reference vaccine should be replaced when necessary.

Potency tests
Potency tests should reflect the activity of the vaccine and should be able to identify vaccines of low potency, which may be of reduced immunogenicity in humans. At the time when the WHO Requirements for recombinant hepatitis B vaccines were published, it was considered that assays that determine the HBsAg content of adjuvanted vaccines would be difficult to standardize. Therefore, it was proposed that immunogenicity in mice should form the basis for determining vaccine potency – by comparing the antibody response induced by the test and by the vaccine reference preparation – and that the specification for potency should be approved by the NRA.

Several manufacturers have since developed and validated in vitro potency tests that are suitable for monitoring the product consistency of their individual vaccines. As a result of the implementation of the in vitro test, the mouse potency
test is no longer being performed by these manufacturers on every final lot. Since the vaccines in question were well established and had been used in millions of individuals, an amendment to the WHO Requirements was published in 1997 (2). This amendment permitted the use of a validated in vitro test to determine vaccine potency as one parameter for monitoring the consistency of production with specifications approved by the NRA.

The in vivo assay should be used to establish the consistency of production of a new hepatitis B vaccine and in vaccine stability studies. In addition, the in vivo potency test should be used to characterize the vaccine after significant changes in the manufacturing process.

**In vivo potency tests**

A suitable quantitative potency test in mice has been developed (3, 4). In brief, groups of 10–20 mice, 5 weeks of age, or in the weight range 17–22 g, are immunized intraperitoneally with a series of at least three dilutions of either the reference or the test vaccine, using a suitable diluent. Some manufacturers use a diluent that contains the same concentration of alum as the vaccine. The strain of mice used for this test must give a suitable dose–response curve with the reference and test vaccines. The concentrations of vaccine tested should be selected to permit the calculation of 50% seroconversion to antibodies against HBsAg. The ED$_{50}$ for both the test vaccine and the reference vaccine should lie within the doses administered. Terminal bleeds are taken after 28 or 42 days, or when an adequate antibody response has developed. Individual sera are assayed for antibodies to HBsAg.

Points that should be considered in establishing such an assay are:

- the strain and sex of the mice used, which must give a suitable dose–response to the reference and test vaccines;
- the number of mice per dilution required to meet the validity criteria of the test;
- the nature and composition of the diluent used to prepare the dilutions of the test vaccine (e.g. containing the adjuvant at the same concentration as used in the vaccine);
- the number of dilutions and appropriate selection of doses to be tested;
- the concentrations of vaccine tested, which should be selected to permit calculation of the dilution giving 50% seroconversion (i.e. ED$_{50}$);
- the assay used to determine the concentration of antibodies to HBsAg in sera (e.g. specificity of monoclonal antibody used);
calculation of the cut-off value (threshold), which is calculated either from the responses of the control group of mice immunized with diluent (e.g. mean optical density of negative control + 2 standard deviations) or using a threshold expressed in mIU/ml as an arbitrary value (e.g. 10 mIU/ml, which is the level indicative of seroprotection in humans); it is important that the choice of cut-off should generate an optimal dose response with the specific dilution range;

- the statistical approach used to analyse the results (e.g. probit);
- interpretation of the results – ED50 or relative potency;
- the establishment of an in-house mouse anti-HBsAg reference serum or panel of high-, medium- and low-titre sera, which can be used to monitor kit performance and assist in the evaluation of new kits or comparison of results from different laboratories (e.g. the manufacturer and the NCL);
- the 95% confidence limits of the potency estimates for each test vaccine, which should fall in the range 33–300%.

In vitro potency tests

Several manufacturers have validated in vitro potency tests based on the determination of HBsAg in dilutions of the vaccine using a commercial detection kit or another method that quantifies the HBsAg antigen content in the vaccine. Two manufacturers have described a method based on an inhibition approach in which dilutions of the vaccine are incubated with a fixed amount of polyclonal anti-HBsAg antibodies, where detection of the unbound antibody is directly related to the amount of HBsAg in the vaccine (5–7). Whichever type of assay is used, the validation studies should show that the assay is suitable for verifying the consistency of production.

In vitro potency tests should be able to distinguish vaccine of low potency, which may include vaccines of low immunogenicity in humans (e.g. lots tested during dose-finding clinical studies) and vaccine samples with artificially reduced potency obtained following incubation for 7 or 15 days at 60 °C or by incubation overnight at 37 °C with 100 ppm of hydrogen peroxide (6, 7). Although vaccines that have been frozen are known to be of low immunogenicity in humans due to an effect on the adjuvant, such vaccines may not necessarily show low potencies in in vitro assays.

When a manufacturer introduces a change in the test method, this must be fully validated according to the guidelines of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and, preferably, according to the test run in parallel with the previous assay. If this is not possible, vaccine lots assessed using the old
method may be used so that any potential variations are detected and managed appropriately.

Important parameters that should be determined during validation include the assessment of both commercial lots and reference vaccine concurrently at optimal dilutions, in order to produce dose–response curves suitable for quantitative analysis by an appropriate statistical method. The statistical validity of the assay should be assessed, the potency of the test relative to the reference vaccine should be estimated, and the assay’s precision (i.e. confidence interval) should be calculated. The 95% confidence limits of the potency estimates for each test vaccine should fall in the range 80–125%. Acceptance criteria for the in vitro assay should be established on the basis of the assay of a suitable number of consecutive final lots.

The vaccine formulation may influence the parameters used in the assay. Therefore, it is recommended that optimization of the assay should be carried out when a new formulation is tested (e.g. when the level of preservative is modified). Each manufacturer should set a specification for the in vitro test that ensures that vaccines that pass this test will also pass the mouse immunogenicity test.

Several factors must be considered when validating an assay, namely:

- the specific reagents and/or type of commercial kit used, where the type of HBsAg used may differ from the vaccine HBsAg (e.g. because of specificities introduced by the vaccine manufacturing process or the expression system for the vaccine HBsAg);
- the adjuvant used in the vaccine and the possible need for a pre-treatment step (e.g. with detergent);
- the reference preparation used (e.g. monovalent HBsAg, combination vaccine);
- the nature of the diluent used to prepare the dilutions of the test and reference vaccine;
- a statistical approach for analysing the results;
- establishment of a test specification based on data from assays on a series of typical production batches of vaccines that pass the mouse immunogenicity test.

The validation of an in vitro potency test should be based on ICH principles (ICH Topic Q 2 CPMP/ICH/381/95) (8) and should include:

- specificity;
- precision (including repeatability, intermediate precision and reproducibility);
- linearity;
range (limits of quantification);
- robustness, which should be documented during assay development.

In vitro assay for antigen quantification of aqueous bulk (non-adjuvanted antigen)

The same in vitro assay used to determine vaccine potency is generally used to determine the HBsAg content of the aqueous bulk. Therefore, it is important to minimize the impact of changes in commercial kits and to use appropriate reference preparations. This reference material could be a representative bulk of known HBsAg protein content, or a highly purified preparation of HBsAg of known protein content stored in single-use aliquots. It is important to note that reference materials based on adjuvanted product are not suitable for use in assays of non-adjuvanted intermediate bulks of HBsAg. Although formulation of final vaccine bulk is generally based on protein content, some manufacturers have chosen to use HBsAg content for this in-process step, and therefore the standardization and monitoring of this assay are critical.

Establishment of product-specific reference

The vaccine potency (in vivo and in vitro) should be assessed against a product-specific reference preparation. The first reference preparation should be established using a vaccine lot that is found to be effective and safe in clinical trials or, alternatively, a vaccine lot that is traceable to a vaccine lot of proven effectiveness and safety. Points to consider when establishing a product-specific reference preparation include:

- source;
- quantity (availability);
- full characterization;
- evaluation with the mouse potency test;
- evaluation with an in vitro potency test;
- stability studies (accelerated degradation and real-time stability);
- establishment of control charts to monitor reference performance.

Replacement of product-specific reference

Standards should be routinely monitored and should be replaced before they begin to show loss of activity. The shelf-life of standards may be longer than the shelf-life of vaccine for routine use, if data to demonstrate the stability of an individual vaccine for this period are available. The shelf-life should be established under the defined storage conditions and maintenance of sterility. A replacement
working standard should be a typical batch of vaccine that is preferably of similar potency to the previous standard.

Points to consider in establishing and replacing a reference vaccine are:

- documentation of the procedure for replacing standards;
- information on product/reference stability and establishment of shelf-life;
- procedure to monitor loss of potency (e.g. trending of relevant values such as changes in dose–response curves, changes of values compared to an internal reference preparation such as a non-adjuvanted stable HBsAg);
- definition of acceptable limits of trended values (e.g. mean initial potency minus 3 standard deviations);
- review of batch record of the new reference vaccine to ensure it complies with the specifications in the marketing authorization;
- calibration of the new reference vaccine against the current reference, using both in vivo and in vitro tests.

Issues relating to potency tests on the hepatitis B component of combination vaccines

Optimization of in vitro assays should be undertaken when they are used with combination vaccines containing HBsAg, since there is evidence that some vaccine components may interfere with such tests (7). If an in vitro assay is not suitable for a particular combination, an in vivo assay should be used. This should be performed at the level of the final bulk.

If a monovalent hepatitis B vaccine is used as a reference in in vivo potency assays of combination vaccines, consideration must be given to the adjuvant effect of whole-cell pertussis and whether the release specification applied to monovalent vaccines is applicable.

References


Appendix 2

Summary protocol for manufacture and control of hepatitis B vaccines

The following protocol is intended for guidance. It indicates the minimum information that should be provided by the manufacturer to the NRA. Information and tests may be added or deleted as necessary, in order to bring the protocol in line with the marketing authorization approved by the NRA. It is thus possible that a protocol for a specific product may differ in detail from the model provided. The essential point is that all relevant details demonstrating compliance with the licence and with the relevant WHO recommendations on a particular product should be given in the protocol submitted.

The section concerning the final product must be accompanied by a sample of the label from the vaccine container and a copy of the leaflet that accompanies it. If the protocol is submitted in support of a request to permit importation, it should also be accompanied by a lot release certificate from the NRA of the country in which the vaccine was produced/released, stating that the product meets national requirements as well as the recommendations in Part A of this document published by WHO.

1. Summary information on the final vaccine lot

International name: ________________________________
Trade name: ________________________________
Batch number(s): ________________________________
Finished product (final vaccine lot) ________________________________
Final vaccine bulk: ________________________________
Type of container: ________________________________
Total number of containers in this batch: ____________________
Number of doses per container: ____________________
Composition (antigen concentration)/volume single human dose: ____________________
Date of last potency test by the manufacturer: ____________________
Date of expiry: ____________________
Storage temperature: ____________________
Product licence (marketing authorization) number: ____________________
Marketing authorization issued by: ____________________
Name and address of manufacturer: ____________________
Name and address of product licence holder (if different from manufacturer): ____________________
2. Production information

Batch number(s) of aqueous bulk(s): ________________________________

Site(s) of manufacture of aqueous bulk(s): ___________________________

Date of manufacture of aqueous bulk: ________________________________

Site of manufacture of finished product
  (final vaccine lot): _____________________________________________

Date of manufacture of finished product (final vaccine lot): _____________

A genealogy of the lot numbers of all vaccine components used in the
formulation of the final product is useful supplementary information.

The following sections are intended for reporting the results of the tests
performed during the production of the vaccine.

3. Starting materials

The information requested below is to be presented on each submission. Full details
on master and working seed lots and cell banks should be provided upon first
submission only and whenever a change has been introduced.

Cell banks

Source of HBsAg (expression system): ________________________________

Master cell bank (MCB) lot number and preparation date: ________________

Population doubling level (PDL) of MCB: ______________________________

Date of approval of protocols indicating compliance
  with the requirements of the relevant monographs
  and the marketing authorization: ________________________________

Manufacturer’s working cell bank (MWCB) lot number
  and preparation date: _________________________________________

Population doubling level (PDL) of MWCB: ___________________________

Date of approval of protocols indicating compliance
  with the requirements of the relevant monographs
  and the marketing authorization: ________________________________

Production cell lot number: _________________________________________

Storage condition: ________________________________________________

Identification of cell substrate

Method: _________________________________________________________

Specification: ____________________________________________________

Date of test: _____________________________________________________

Result: __________________________________________________________
Nature and concentration of antibiotics or selecting agent(s) used in production cell culture maintenance medium: ____________________________

Identification and source of starting materials used in preparing production cells, including excipients and preservatives (particularly any materials of human or animal origin, e.g. albumin, serum): ____________________________

4. Fermentation

**Mammalian cells**: *provide information on cells corresponding to each single harvest*

Ratio or proportion of control cell cultures to production cell cultures: ____________________________

Volume of control cells: ____________________________

Period of observation of cultures: ____________________________

Percentage rejected for nonspecific reasons: ____________________________

Result: ____________________________

**Haemadsorbing viruses**

Type(s) of red blood cells: ____________________________

Storage time and temperature of red blood cells: ____________________________

Incubation time and temperature of red blood cells: ____________________________

Percentage of cultures tested: ____________________________

Date when test started: ____________________________

Date when test ended: ____________________________

Result: ____________________________

**Tests on supernatant fluids for other adventitious agents (if relevant)**

Date of sampling from production cell cultures: ____________________________

**Type of simian cells**: ____________________________

Quantity of sample inoculated: ____________________________

Incubation temperature: ____________________________

Date when test started: ____________________________

Date when test ended: ____________________________

Percentage of viable culture at the end: ____________________________

Result: ____________________________

**Type of human cells**: ____________________________

Quantity of sample inoculated: ____________________________

Incubation temperature: ____________________________

Date when test started: ____________________________
Date when test ended: ________________________________
Percentage of viable culture at the end: ________________________________
Result: ________________________________

Type(s) of other diploid cells: ________________________________
Quantity of sample inoculated: ________________________________
Incubation temperature: ________________________________
Date when test started: ________________________________
Date when test ended: ________________________________
Percentage of viable culture at the end: ________________________________
Result: ________________________________

Type(s) of other diploid cells: ________________________________
Quantity of sample inoculated: ________________________________
Incubation temperature: ________________________________
Date when test started: ________________________________
Date when test ended: ________________________________
Percentage of viable culture at the end: ________________________________
Result: ________________________________

**Mammalian and yeast cells**

**Bacteria and fungi**

Method: ________________________________
Media and temperature of incubation: ________________________________
Volume inoculated: ________________________________
Date of inoculation: ________________________________
Date of end of observation: ________________________________
Result: ________________________________

**Mycoplasmas (for mammalian cells)**

Method: ________________________________
Media: ________________________________
Volume inoculated: ________________________________
Date when test started: ________________________________
Date when test ended: ________________________________
Result: ________________________________

**5. Single harvests (or pools)**

Batch number(s): ________________________________
Date of inoculation: ________________________________
Date of harvesting: __________________________________________________________________________
Volume(s) of fermentation paste, storage temperature, storage time and approved storage period: __________________________________________________________________________

**Culture purity or sterility for bacteria and fungi**
- Method: __________________________________________________________________________
- Media and temperature of incubation: __________________________________________________________________________
- Volume inoculated: __________________________________________________________________________
- Date when test started: __________________________________________________________________________
- Date when test ended: __________________________________________________________________________
- Result: __________________________________________________________________________

**Plasmid retention**
- Method: __________________________________________________________________________
- Specification: __________________________________________________________________________
- Date of test: __________________________________________________________________________
- Result: __________________________________________________________________________

*In addition, the following tests should be conducted if mammalian cells are used.*

**Adventitious agents**
- Method: __________________________________________________________________________
- Specification: __________________________________________________________________________
- Date of test: __________________________________________________________________________
- Result: __________________________________________________________________________

**Mycoplasmas**
- Method: __________________________________________________________________________
- Media: __________________________________________________________________________
- Volume inoculated: __________________________________________________________________________
- Date when test started: __________________________________________________________________________
- Date when test ended: __________________________________________________________________________
- Result: __________________________________________________________________________

**Mycobacterium spp. (if applicable)**
- Method: __________________________________________________________________________
- Media and temperature of incubation: __________________________________________________________________________
- Volume inoculated: __________________________________________________________________________
- Date when test started: __________________________________________________________________________
- Date when test ended: __________________________________________________________________________
- Result: __________________________________________________________________________
Reverse transcriptase assay
Method: __________________________________________________________
Specification: _____________________________________________________
Date of test: _______________________________________________________
Result: __________________________________________________________

6. Control of aqueous bulk (purified antigen)
Batch number(s) of purified bulk: _______________________________________
Date(s) of purification(s): _____________________________________________
Volume(s), storage temperature, storage time
and approved storage period: ___________________________________________

Purity (add PAGE photographs if applicable)
Method: ___________________________________________________________
Specification: _______________________________________________________
Date of test: _______________________________________________________
Result: ___________________________________________________________

Protein content
Method: _____________________________________________________________
Specification: _______________________________________________________
Date of test: _______________________________________________________
Result: ___________________________________________________________

HBsAg antigen content/identity
Method: _____________________________________________________________
Specification: _______________________________________________________
Date of test: _______________________________________________________
Result: ___________________________________________________________

Ratio of HBsAg antigen: protein content
Specification: _______________________________________________________
Result: ___________________________________________________________

Bacteria and fungi
Method: _____________________________________________________________
Media and temperature of incubation: _________________________________
Volume inoculated: _________________________________________________
Date when test started: ______________________________________________
Date when test ended: _______________________________________________
Result: ___________________________________________________________
### Lipid
- **Method:**
- **Specification:**
- **Date of test:**
- **Result:**

### Carbohydrate
- **Method:**
- **Specification:**
- **Date of test:**
- **Result:**

### Potential hazards, e.g. residual chemical(s) (if relevant)
- **Method:**
- **Specification:**
- **Date of test:**
- **Result:**

### Residual DNA (if applicable)
- **Method:**
- **Specification:**
- **Date of test:**
- **Result:**

### Bacterial endotoxins
- **Method:**
- **Specification:**
- **Date of test:**
- **Result:**

### Albumin content (if mammalian cells and animal serum are used for production)
- **Method:**
- **Specification:**
- **Date of test:**
- **Result:**

### Tests on adjuvant bulk
#### Adjuvant or mineral vehicle concentration (if applicable)
- **Method:**
- **Specification:**
- **Date of test:**
- **Result:**
Degree of adsorption (if applicable)
Method: ________________________________
Specification: ________________________________
Date of test: ________________________________
Result: ________________________________

Bacteria and fungi
Method: ________________________________
Media and temperature of incubation: ________________________________
Volume inoculated: ________________________________
Date of start of test: ________________________________
Date of end of test: ________________________________
Result: ________________________________

pH
Method: ________________________________
Specification: ________________________________
Date of test: ________________________________
Result: ________________________________

7. Final vaccine bulk
Batch number(s) of aqueous bulk: ________________________________
Formulation date: ________________________________
Batch number(s) of all components used during adjuvant formulation: ________________________________
Volume, storage temperature, storage time and approved storage period: ________________________________

Bacteria and fungi
Method: ________________________________
Media and temperature of incubation: ________________________________
Volume inoculated: ________________________________
Date of start of test: ________________________________
Date of end of test: ________________________________
Result: ________________________________

Identity
Method: ________________________________
Specification: ________________________________
Date of test: ________________________________
Result: ________________________________
Adjuvant or mineral vehicle concentration (if applicable)

Method: ________________________________
Specification: __________________________
Date of test: ____________________________
Result: ________________________________

Degree of adsorption (if applicable)

Method: ________________________________
Specification: __________________________
Date of test: ____________________________
Result: ________________________________

pH

Method: ________________________________
Specification: __________________________
Date of test: ____________________________
Result: ________________________________

Preservative

Method: ________________________________
Specification: __________________________
Date of test: ____________________________
Result: ________________________________

Potency test: in vivo assay (if applicable)

Species, strain, sex and weight specifications: __________________
Dates of vaccination, bleeding: __________________________
Date of assay: ________________________________
Batch number of reference vaccine and assigned potency: ____________
Vaccine doses (dilutions) and number of animals responding at each dose: ________________
ED$_{50}$ of reference and test vaccine: __________________________
Potency of test vaccine vs. reference vaccine with 95% fiducial limits of mean: ________________
Validity criteria: __________________

8. Final vaccine lot

Batch number: ________________________________
Date of filling: ________________________________
Type of container: ____________________________
Filling volume: ________________________________
Number of containers after inspection: ________________
Appearance
Method: _____________________________
Specification: _____________________________
Date of test: _____________________________
Result: _____________________________

Identity
Method: _____________________________
Specification: _____________________________
Date of test: _____________________________
Result: _____________________________

Bacteria and fungi
Method: _____________________________
Media and temperature of incubation: _____________________________
Volume inoculated: _____________________________
Date when test started: _____________________________
Date when test ended: _____________________________
Result: _____________________________

pH
Method: _____________________________
Specification: _____________________________
Date of test: _____________________________
Result: _____________________________

Osmolality
Method: _____________________________
Specification: _____________________________
Date of test: _____________________________
Result: _____________________________

Preservatives (if applicable)
Method: _____________________________
Specification: _____________________________
Date of test: _____________________________
Result: _____________________________

Pyrogenic substances
Method: _____________________________
Specification: _____________________________

WHO Expert Committee on Biological Standardization
Sixty-first report

Date of test: ____________________________
Result: ____________________________

**Adjuvant content**

Method: ____________________________
Specification: ____________________________
Date of test: ____________________________
Result: ____________________________

**Protein content (or calculated value)**

Method: ____________________________
Specification: ____________________________
Date of test: ____________________________
Result: ____________________________

**Degree of adsorption (if applicable)**

Method: ____________________________
Specification: ____________________________
Date of test: ____________________________
Result: ____________________________

**Potency:**

*In vitro assay*

Method: ____________________________
Batch number of reference vaccine and assigned potency: _________
Specification: ____________________________
Date of assay: ____________________________
Result: ____________________________

*If an in vivo assay is used (may be performed at final bulk stage)*

Species, strain, sex and weight specifications: ____________________________
Number of mice tested: ____________________________
Dates of vaccination, bleeding: ____________________________
Date of assay: ____________________________
Batch number of reference vaccine and assigned potency: _________
Vaccine doses (dilutions) and number of animals responding at each dose: ____________________________
$ED_{50}$ of reference and test vaccine: ____________________________
Potency of test vaccine vs reference vaccine with 95% fiducial limits of mean: ____________________________
Validity criteria: ____________________________
Date of start of period of validity: ____________________________
**General safety (unless deletion authorized)**

**Test in mice**
- Number of mice tested: ____________________________
- Volume and route of injection: ____________________________
- Date of injection: ____________________________
- Date of end of observation: ____________________________
- Specification: ____________________________
- Result: ____________________________

**Test in guinea-pigs**
- Number of guinea-pigs tested: ____________________________
- Volume and route of injection: ____________________________
- Date of injection: ____________________________
- Date of end of observation: ____________________________
- Specification: ____________________________
- Result: ____________________________

**Freezing-point (if applicable)**
- Method: ____________________________
- Specification: ____________________________
- Date of test: ____________________________
- Result: ____________________________
Appendix 3

Model certificate for the release of recombinant hepatitis B vaccine by a national regulatory authority

This certificate is to be provided by the national regulatory authority of the country where the vaccines have been manufactured, upon request of the manufacturer

Certificate no. ____________________

LOT RELEASE CERTIFICATE

The following lot(s) of recombinant hepatitis B vaccine produced by ____________________ 1 in ____________________, 2 whose numbers appear on the labels of the final containers, meet all national requirements 3 and Part A 4 of the WHO Recommendations to assure the quality, safety and efficacy of recombinant hepatitis B vaccines ( ____________________ ) 5, and comply with Good manufacturing practices for pharmaceutical products: main principles 6 and Good manufacturing practices for biological products 7.

As a minimum, this certificate is based on examination of the summary protocol of manufacturing and control.

<table>
<thead>
<tr>
<th>Final lot no.</th>
<th>No. of released human doses in this final vaccine lot</th>
<th>Expiry date</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The Director of the national regulatory authority (or authority as appropriate):

Name (typed) __________________________________________
Signature __________________________________________
Date ________________________________________________

---

1 Name of manufacturer.
2 Country of origin.
3 If any national requirements are not met, specify which one(s) and indicate why release of the lot(s) has nevertheless been authorized by the national regulatory authority.
4 With the exception of provisions on distribution and shipping, which the national regulatory authority may not be in a position to assess.
Annex 5

Recommendations to assure the quality, safety and efficacy of live attenuated yellow fever vaccines


Abbreviations

1. Introduction

2. General considerations

Part A. Manufacturing recommendations

A.1 Definitions
A.2 Certification of the substrain of 17D virus for use in vaccine production
A.3 General manufacturing recommendations
A.4 Control of source materials
A.5 Control of vaccine production
A.6 Filling and containers
A.7 Control tests on final lot
A.8 Records
A.9 Retained samples
A.10 Labelling
A.11 Distribution and shipping
A.12 Stability, storage and expiry date

Part B. Nonclinical evaluation of live attenuated yellow fever vaccines

B.1 Characterization of a new candidate yellow fever vaccine
B.2 Immunogenicity and other pharmacodynamic studies
B.3 Toxicity assessment

Part C. Clinical evaluation of live attenuated yellow fever vaccines

C.1 General considerations
C.2 Safety and immunogenicity studies
C.3 Post-marketing studies and surveillance

Part D. Recommendations for national regulatory authorities

D.1 General
D.2 Release and certification

Acknowledgements

References
Recommendations published by WHO are intended to be scientific and advisory. Each of the following sections constitutes guidance for national regulatory authorities (NRAs) and for manufacturers of biological products. If an NRA so desires, these recommendations may be adopted as definitive national requirements, or modifications may be justified and made by the NRA. It is recommended that modifications to these recommendations be made only on condition that the modifications ensure that the vaccine is at least as safe and efficacious as that prepared in accordance with the recommendations set out below. The parts of each section printed in small type are comments for additional guidance intended for manufacturers and NRAs, which may benefit from these details.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AEFI</td>
<td>adverse events following immunization</td>
</tr>
<tr>
<td>ALV</td>
<td>avian leukosis virus</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>C</td>
<td>capsid (structural protein)</td>
</tr>
<tr>
<td>CMC</td>
<td>carboxymethyl cellulose</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>E</td>
<td>envelope (structural protein)</td>
</tr>
<tr>
<td>EPI</td>
<td>Expanded Programme on Immunization</td>
</tr>
<tr>
<td>FNV</td>
<td>French neurotropic vaccine</td>
</tr>
<tr>
<td>GMT</td>
<td>geometric mean titre</td>
</tr>
<tr>
<td>HAI</td>
<td>haemagglutination inhibition</td>
</tr>
<tr>
<td>HI</td>
<td>haemagglutination inhibition</td>
</tr>
<tr>
<td>IgM</td>
<td>immunoglobulin M</td>
</tr>
<tr>
<td>IU</td>
<td>International Unit</td>
</tr>
<tr>
<td>LD&lt;sub&gt;50&lt;/sub&gt;</td>
<td>median lethal dose</td>
</tr>
<tr>
<td>LNI</td>
<td>log10 neutralization index</td>
</tr>
<tr>
<td>M</td>
<td>membrane (structural protein)</td>
</tr>
<tr>
<td>MMR</td>
<td>measles–mumps–rubella</td>
</tr>
<tr>
<td>NAT</td>
<td>nucleic acid amplification technique</td>
</tr>
<tr>
<td>NISBC</td>
<td>National Institute for Biological Standards and Control</td>
</tr>
<tr>
<td>NRA</td>
<td>national regulatory authority</td>
</tr>
<tr>
<td>NS</td>
<td>non-structural (protein)</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PFU</td>
<td>plaque-forming unit</td>
</tr>
<tr>
<td>PRNT</td>
<td>plaque-reduction neutralization test</td>
</tr>
<tr>
<td>PS</td>
<td>pig kidney epithelial cells (latently infected with swine fever virus)</td>
</tr>
<tr>
<td>RCD</td>
<td>reverse cumulative distribution</td>
</tr>
</tbody>
</table>
1. Introduction

Requirements for yellow fever vaccine were first formulated in 1958, by the WHO Study Group on Requirements for Yellow Fever Vaccine and Requirements for Cholera Vaccine, and were published as Requirements for yellow fever vaccine (1). The Requirements embodied recommendations made by the WHO Expert Committee on Yellow Fever Vaccine at its first meeting (2), and they applied to vaccine prepared from a suitable strain of yellow fever virus. The vaccine was intended to be given by subcutaneous injection. Conformity with these Requirements has been the basis for WHO approval of yellow fever vaccine used for vaccination and revaccination against yellow fever in connection with certification for the purposes of international travel (3). Such approval has been given only to vaccine prepared using seed derived from the 17D strain of yellow fever virus. Yellow fever continues to be the only disease for which a certificate of vaccination is required for entry into some countries, and the update of the International Health Regulations (4) increased attention to the need for such certificates. The Requirements have also been used by national regulatory authorities (NRAs) for the control and approval of yellow fever vaccine used in national immunization programmes.

In 1969, at its twenty-second meeting, the WHO Expert Committee on Biological Standardization agreed that developments in virology in general, and in the manufacture and control of yellow fever vaccine in particular, warranted a revision of the existing Requirements, with due consideration of their national and international applications (5). In 1975, at its twenty-seventh meeting, the Committee formulated revised Requirements for yellow fever vaccine (6). Much experience was gained with the preparation of yellow fever vaccine after 1975, and a further revision of the Requirements was approved by the Committee in 1995 (7).

A collaborative study to assess the suitability of a candidate International Standard for yellow fever vaccine indicated that the use of a standard for measuring potency, which has been assigned an arbitrary unitage in International Units (IU), would markedly improve the agreement between the results of different laboratories (8). The First International Standard for yellow fever vaccine with an assigned potency of 10^{1.5} IU per ampoule was established in 2003 (9). A proposal...
to amend the Requirements for yellow fever vaccine so that the potency of such vaccines could be expressed in IU per dose, and the dose recommended for use in humans should be not less than 3.0 log₁₀ IU with no upper limit on the quantity of virus in a dose, was approved by the Committee in 2008 (10). The availability of an International Standard for yellow fever vaccine with an assigned potency in IU, such that assay in mice and expression of virus titres in median lethal dose (LD₅₀) are not required, also impacts on other sections of the Requirements established in 1995 (7).

In 2008, the Committee recommended that the Requirements for yellow fever vaccines be reviewed, as it was more than 10 years since they had been published and sections on nonclinical and clinical evaluation for new candidate yellow fever vaccines were required. To facilitate this process, WHO convened a meeting of experts, regulatory professionals and other stakeholders in Geneva in May 2009, to discuss the scientific basis for the present revision of the Requirements and to develop revised Recommendations for yellow fever vaccines (11).

The scope of the current Recommendations encompasses live attenuated yellow fever vaccines derived from strain 17D, including 17D-204 and 17DD substrains.

This document should be read in conjunction with the relevant WHO guidelines, including those on nonclinical (12) and clinical evaluation (13) of vaccines.

2. General considerations

The yellow fever virus is small (50 nm) and consists of a nucleocapsid with core protein (13 kDa) containing single-stranded, positive-sense RNA surrounded by a lipoprotein envelope (14). The lipoprotein envelope contains two proteins – a small membrane protein (8 kDa) and an envelope glycoprotein (53 kDa), which is the major target of neutralizing antibodies and has type- and group-specific antigenic determinants. Wild-type yellow fever viruses have genomes of similar length but vary depending on the size of the 3′ non-coding region (15, 16). On the basis of sequence analysis, wild-type yellow fever virus strains have been classified into at least seven genotypes: five in Africa and two in South America. The genotypic variation is not accompanied by significant antigenic differences across strains and there is a single serotype (17).

The genome of the yellow fever virus strain from which all 17D vaccines are derived has been completely sequenced and has been found to contain 10,862 nucleotides, which encode three structural and seven non-structural proteins (18). There are two substrains in use today for the manufacture of 17D vaccine, namely 17D-204 and 17DD. 17D-213 is a derivative of 17D-204 that has gained
a glycosylation site in the E protein but differs significantly in phenotype from 17D-204. It is sometimes considered to be a substrain of 17D and sometimes referred to as 17D-213. Genomic sequencing has been reported for many of the yellow fever vaccine viruses and their seeds that are currently used by different manufacturers. These studies show that there are very few nucleotide and amino acid differences between the vaccine strains. The yellow fever vaccine strains that have been and are being used for vaccine manufacture, and their history, are outlined in Appendix 1.

Yellow fever is a viral haemorrhagic fever that is endemic to 32 countries in Africa and 13 countries in Central and South America (19).

In 1900, a commission headed by the American physician Walter Reed confirmed that the disease was transmitted from human to human by the mosquito *Aedes aegypti*, a hypothesis proposed earlier by the Cuban physician Carlos Finlay in 1881 (20). There are two epidemiological patterns of yellow fever virus transmission: the urban cycle and the forest cycle (also known as the jungle or sylvan cycle). The two patterns of transmission lead to a clinically identical disease. In the Americas, the yellow fever virus circulates by means of an endemic forest cycle that results in as many as several hundred reports per year of infection, primarily in non-immune forest workers, with occasional reports of isolated cases of urban yellow fever. In Africa, the virus circulates by means of both urban and forest cycles and periodically breaks out of its endemic pattern to infect large numbers of non-immune persons in the course of major epidemics (21).

The case-fatality rate of yellow fever can reach as high as 20–80% in severely ill patients who are hospitalized (22). Case-fatality rates are highest among young children and the elderly. There are no antiviral drugs for any flavivirus infection, including yellow fever, so the availability of vaccines is important for both resident populations and travellers.

When 17D vaccine was first used in the late 1930s and early 1940s, some problems were observed that were associated with under- or over-attenuation of the 17D strain on passage. These problems were resolved by the establishment of a virus seed lot system in 1945. As of 2009, more than 500 million doses of 17D vaccine had been administered (23), so a large amount of information is available on vaccine safety. This vaccine has been shown to be very effective for the control of yellow fever both during outbreaks and between epidemics. In 1990, the Global Advisory Group of the Expanded Programme on Immunization (EPI) recommended that all countries at risk of yellow fever should incorporate the vaccine in their routine immunization programmes. In Africa, 22 countries have introduced yellow fever vaccine in routine childhood immunization. Routine vaccination coverage in countries at risk in Africa increased from 16% in 2000 (eight countries) to 43% in 2008. In the Americas, coverage rose from 64% to 91% (19). In this regard, it is of note that the limited data on vaccination...
of individuals with immunosuppression associated with infection with HIV suggest that seroconversion is reduced without an increase in adverse events following immunization (AEFI) (24).

Serious adverse reactions that have been reported to be associated with the administration of 17D yellow fever vaccine and that are of particular note include the following:

- Hypersensitivity reactions, including anaphylaxis, are believed to be associated with egg protein, owing to the vaccine being grown in embryonated chicken eggs. However, gelatine used by some manufacturers may be implicated in some hypersensitivity reactions.

- Yellow fever vaccine-associated neurological disease (YEL-AND) is a term recently introduced to define neurological AEFIs that have occurred in temporal association with yellow fever vaccination since 2000 (25). Encephalitis following 17D vaccination in vaccinees of any age was first described in the 1940s (26). The incidence rate was dramatically reduced to background levels after introduction of the seed lot system for manufacture of 17D vaccines. However, in the 1950s, there were several individual case reports describing a self-limited encephalitis in infants and very young children that occurred in temporal association with 17D vaccines manufactured in accordance with the seed lot system (see section A.4.2.1). With one exception, these children recovered fully with no sequelae. Nevertheless, these reports led to the recommendation by WHO that infants aged 6 months and below should not be vaccinated (17). The adoption of this recommendation and possible unknown factors led to the virtual elimination of post-vaccinal encephalitis by the mid-1960s. However, since 2000, there have been rare case reports of a variety of neurological AEFIs in 17D vaccinees of all ages, and particularly in the elderly (25). Rates of YEL-AND varied in different studies undertaken in different populations, but were observed to range from 0.19 to 0.8 per 100 000 doses in studies in Europe and the United States of America (USA) (25, 27). Vaccines of both 17D-204 and 17DD substrains have been associated with YEL-AND.

- A total of 51 cases of yellow fever vaccine-associated viscerotropic disease (YEL-AVD) had been identified up to May 2009 (23). The estimated reporting rate is between 0.004 and 0.4 per 100 000 doses, with a case-fatality rate of up to 64%. All the reported cases occurred after the primary dose (23). The published index case is from Brazil in 1975 (28). The mechanism(s) responsible for the clinical picture of YEL-AVD, which can vary from multi-organ system failure without much evidence of hepatitis to a fulminant hepatitis resembling the
disease yellow fever, is currently unknown (29–32). Available data suggest that YEL-AVD is related to individual, genetically determined and currently unknown host factors, rather than to the vaccine virus itself. Molecular and animal studies performed to date provide no evidence that 17D vaccine virus mutations have contributed to YEL-AVD (33, 34).

In 2007, a cluster of five YEL-AVD cases was reported after a yellow fever mass vaccination campaign in Peru, with four fatal cases that were confirmed virologically and clinically among 42,000 vaccinees who received vaccine from the same lot. This was the first (and so far the only) occasion that a cluster of YEL-AVD cases has been observed in association with a particular lot of vaccine, and it remains unexplained. No quality issues were identified in the manufacture of the vaccine, and the characterization of the working seed and batch records were satisfactory. There were no reported problems from nine batches prepared from the same final bulk as the lot associated with YEL-AVD. The virus isolated from one of the individuals was sequenced and found to be vaccine virus with no evidence that it had mutated (35, 36). An expert panel that was convened to investigate the reports found no features of the vaccine lot that would explain the cluster of cases (35), even though deaths were due to extensive replication of vaccine virus in multiple organs. No difference was identified between the quality of this lot and that of other lots of vaccine, so it has been concluded that cofactors must have led to these cases of YEL-AVD.

The rarity of YEL-AVD cases and the limited number of clinical samples make it difficult to substantiate hypotheses regarding the underlying pathological mechanisms. One potential hypothesis proposes a disconnection between the signalling of innate immune response and the timely activation of the adaptive immune response. Thus, future work that may lead to a more detailed understanding of the immune response induced by the vaccine may help to explain YEL-AVD pathology. Thus far, risk factors that may be associated with the development of YEL-AVD include age (60 years and above) and a history of thymus disease or ablation.

Between 2007 and 2009, three cases of encephalitis in neonates (aged 10 days to 5 weeks) were reported, in which infection in the infants appeared to have resulted from transmission of yellow fever vaccine virus from their recently vaccinated mothers through breastfeeding (37, 38). The onset of symptoms in those infants ranged from 8 to 25 days after maternal vaccination. One of the three cases was confirmed to be vaccine-associated, by detection of vaccine virus RNA in the cerebrospinal fluid (CSF) of the infant (37). Maternal breast milk was not tested for evidence of vaccine virus in any of the three cases. Direct blood-to-blood transmission, through a break in the maternal areola and the mucosa of the infant’s mouth, was thought to be the possible mode of infection. However,
no examination for possible breast lesions was made in any of the cases. These reports are in accordance with the known risk of encephalitis after vaccination of infants under 6 months of age. Based on these case reports, the potential risk of transmission of yellow fever vaccine virus from vaccinated mothers to breastfeeding infants was recently reviewed by the WHO Global Advisory Committee on Vaccine Safety, which concluded that further research is needed to quantify the potential risk, including the possibility of transmission through breast milk. Such studies might include testing breast milk from vaccinated mothers for the presence of vaccine virus and testing their infants for evidence of seroconversion to the vaccine virus. The committee also noted that the risk of potential transmission may vary according to whether mothers are primary vaccinees or have previously been vaccinated, and the age of the infant when exposed (39).

The first immunoglobulin M (IgM)-confirmed transmission of yellow fever vaccine virus through transfusion of blood donated by recently vaccinated military personnel in the USA was described in 2009 (40). Serological evidence of infection was confirmed in three out of five transfusion recipients, though no adverse events or clinical illness attributable to the infection were reported. This documented finding supports the current widely existing recommendations (previously based on a theoretical risk of vaccine virus transmission) that yellow fever vaccine recipients should defer donating blood products for a period (generally 2 weeks) after vaccination.

It is important to ensure that new master or working seeds are confirmed to exhibit levels of neurotropism and viscerotropism that are comparable with those documented for available 17D vaccines. Owing to the lack of suitable animal models for viscerotropic disease, much weight is currently placed on monkey neurovirulence studies, which have a long history. The relevant safety test, performed on monkeys, is therefore retained in these revised Recommendations.

There have been investigations into alternative animal models. A hamster model has been developed that shows viscerotropic disease (41). However, most wild-type strains (which need to be adapted to hamsters) and viruses from YEL-AVD cases do not show viscerotropic disease in this model. Another study reported results of a mouse model for studying viscerotropic disease caused by yellow fever virus infection, which may have some potential as a small-animal model for yellow fever virus (42). The applicability of these models will have to be established before they can be considered for use in the qualification of virus seeds (see Part B).

The thermostability test (see section A.7.4) is undertaken to demonstrate consistency of production and not as a predictive value of real-time stability (43). At the end of the incubation period, the geometric mean infectious titre in the incubated final containers should not have decreased by more than $1.0 \log_{10}$ IU but there is no requirement for the minimum specification to be met.
Part A. Manufacturing recommendations

A.1 Definitions

A.1.1 International name and proper name

The international name should be “live attenuated yellow fever vaccine”. The proper name should be the equivalent of the international name in the language of the country of origin.

The use of the international name should be limited to vaccines that satisfy the recommendations formulated below.

A.1.2 Descriptive definition

Yellow fever vaccine should consist of a freeze-dried preparation of viable, attenuated yellow fever virus (*Flavivirus hominis*, 17D strain). The preparation should satisfy all the recommendations formulated below.

A.1.3 International Standards

An International Standard for yellow fever vaccine is available from the National Institute for Biological Standards and Control (NIBSC), Potters Bar, England. This material is for use in the calibration of working reference materials for yellow fever vaccine, which are included in each potency test, so that the potency of vaccines is expressed in IU/dose.

NIBSC distributes the International Reference Preparation of anti-yellow-fever serum. Such a preparation is needed as a basis for comparison of antibody responses in the monkey neurovirulence test, and may also be used in antibody assays of clinical trial sera. Additionally, a non-immune control serum is available. These preparations are monkey sera.

WHO reference virus 168-73 is available from NIBSC (see Appendix 2).

A.1.4 Terminology

The definitions given below apply to the terms as used in these Recommendations. They may have different meanings in other contexts.

Adventitious agents: contaminating microorganisms, including bacteria, fungi, mycoplasmas and endogenous and exogenous viruses, that have been unintentionally introduced.

Final bulk: the material prepared from one or more single harvests in the container from which the final containers are filled.

Final lot: a collection of sealed final containers of finished vaccine that are homogeneous with respect to the risk of contamination during filling and freeze-drying. All final containers must, therefore, have been filled from a single container of final bulk in one working session and lyophilized under standardized conditions in a common chamber.
**International Unit (IU):** an IU is a unit of measurement of potency for the yellow fever vaccine, based on the determination of the infectivity of a virus preparation resulting in plaque formation in a suitable tissue culture monolayer in parallel with an accepted working standard, calibrated in IU against the International Standard for yellow fever vaccine.

**Single harvest:** a quantity of virus suspension, derived from tissues of the same origin that were inoculated with the same working seed lot, that has been collected and processed in a single production run.

**Specific-pathogen-free (SPF):** specific-pathogen-free refers to animals that have been shown by the use of appropriate tests to be free from specified pathogenic microorganisms, and also to eggs derived from SPF birds (44, 45).

**Virus master seed lot:** a quantity of virus suspension that has been processed at the same time to assure a uniform composition and that has been characterized to the extent necessary to support development of the virus working seed lot. The characterized virus master seed lot is used for the preparation of virus working seed lots.

**Virus working seed lot:** a quantity of virus of uniform composition, fully characterized and only one passage from a virus master seed lot. The virus working seed lot is used for inoculating embryonated chicken eggs in the preparation of vaccine.

**WHO primary seed virus (213-77):** a quantity of virus suspension of uniform composition produced for WHO by the Robert Koch Institute and available to manufacturers for use in the preparation of a virus master seed lot.

### A.2 Certification of the substrain of 17D virus for use in vaccine production

Currently used substrains 17D-204 and 17DD have a well-documented passage history (see Appendix 1) and safety records, from nonclinical and clinical studies. Any new candidate 17D virus to be used as a master seed for production would require supporting data to qualify it for use. Virus seed lots that have been certified previously can be used. A yellow fever virus primary seed (213-77) is available from WHO on request (previously known as “WHO master seed”) (46). Parts B and C of this document provide recommendations for evaluating new candidate 17D vaccine viruses.

Only seed lots derived from viruses that are approved by the NRA should be used in the production of yellow fever vaccines.

### A.3 General manufacturing recommendations

The general manufacturing recommendations for manufacturing establishments contained in the WHO good manufacturing practices: main principles for pharmaceutical products (47) and the Good manufacturing practices for biological
products (48) should apply to establishments manufacturing yellow fever vaccine. Staff directly involved with the production and testing of yellow fever vaccine should be shown to be immune to yellow fever.

A.4 Control of source materials

A.4.1 Eggs used for seed virus growth and vaccine production

Virus for the preparation of virus master and working seed lots and all vaccine production should be grown in embryonated chicken eggs from a closed SPF flock monitored by methods approved by the NRA or the national animal health authority.

All chickens are bled when an SPF flock is established, and thereafter a percentage of the birds are bled at specified intervals to detect exposure of the flock to microbes with potential to cause quality failure in assessments for adventitious agents. In some countries, SPF flocks are monitored on a weekly basis for quality control. The sera are screened for antibodies to the relevant pathogens. The pathogens may also be detected in the flocks by culture or other detection methods, including polymerase chain reaction (PCR). Any chicken in an SPF flock that dies should be investigated, in order to determine the cause of death.

Microbes of interest in flock husbandry may vary by geographical region but should include as a minimum the following: avian adenoviruses, avian encephalomyelitis virus, avian infectious bronchitis viruses, avian infectious laryngotracheitis virus, avian leukosis viruses (ALVs), avian nephritis virus, avian orthoreoviruses, avian reticuloendotheliosis virus, chicken anaemia virus, egg drop syndrome virus, fowl pox virus, infectious bursal disease viruses, influenza A viruses, Marek disease virus, Newcastle disease virus, Mycobacterium avium, Mycoplasma gallisepticum, Mycoplasma synoviae, Salmonella gallinarum, Salmonella pullorum, Salmonella species and Haemophilus paragallinarum.

The flock must not have been vaccinated with live Newcastle disease virus vaccine. In addition, flocks should not be receiving any chemotherapeutic agents (e.g. antimicrobial agents and coccidiostats). It is also recommended that eggs be obtained from young hens.

A.4.2 Yellow fever virus

The substrain of 17D vaccine virus used in the production of vaccine should be certified as described in section A.2.

A.4.2.1 Virus seed lot system

The production of vaccine should be based on the virus master seed lot and virus working seed lot system.
Virus seed lots should be stored in a dedicated temperature-monitored freezer at a temperature that ensures stability, namely less than –60 °C. In some laboratories, the virus master and working seed lots are stored in more than one location.

The virus master and working seed lots must not contain any human protein or added serum or antibiotics.

The virus master and working seed lots should be free of ALVs, mycoplasmas or other adventitious agents, as shown by suitable tests (see sections A.4.2.2.3 and A.4.2.2.4).

The inoculum for infecting eggs used in the production of vaccine should be from a virus working seed lot without intervening passage, in order to ensure that no vaccine is manufactured that is more than one passage removed from a seed lot that has passed all safety tests.

### A.4.2.2 Tests on virus master and working seeds

#### A.4.2.2.1 Identity

Each virus master and working seed lot should be identified as yellow fever virus, by immunological assay or by molecular methods and comparison to an appropriate published 17D vaccine virus. An identity test should be performed on at least one container from each virus master and working seed lot.

#### A.4.2.2.2 Genotype characterization

For any new virus master and working seed, it is recommended that the first three consecutive consistency vaccine lots be analysed for consensus sequence changes from the seed virus (total genome sequence). The sequence results should be used to demonstrate the consistency of the production process. Routine sequence analysis of final bulk vaccine is not recommended.

#### A.4.2.2.3 Tests for bacteria, fungi and mycoplasmas

Each virus master and working seed lot should be tested for bacterial, fungal and mycoplasmal contamination by appropriate tests, as specified in Part A, sections 5.2 (49) and 5.3 (50) of General requirements for the sterility of biological substances, or by a method approved by the NRA.

Nucleic acid amplification techniques (NATs) alone or in combination with cell culture, with an appropriate detection method, may be used as an alternative to one or both of the compendial mycoplasma detection methods, after suitable validation and agreement from the NRA (51).

#### A.4.2.2.4 Tests for adventitious agents

Each virus master and working seed lot should be tested for ALVs and other adventitious agents relevant to the passage history of the seed virus. In addition,
each virus working seed lot should be tested in both cells and eggs for the other adventitious agents.

Neutralization of yellow fever virus is necessary for many tests because the virus is cytopathogenic. Where antisera are used to neutralize yellow fever virus, the antigen used to generate the antisera should be produced in cell cultures (other than those derived from chickens) and should be free from extraneous agents. After neutralization of the yellow fever virus by hyperimmune antibody preparation, the virus pool should be inoculated on cell cultures of human cells, monkey cells and chicken cells. Following inoculation, the cell cultures should be observed microscopically for cytopathic changes. At the end of the observation period, the cells should be tested for haemadsorbing viruses. The cell cultures, the method of incubation and the period of observation should be approved by the NRA. A specific monoclonal antibody may be used instead of a hyperimmune polyclonal serum.

Each virus master or working seed lot should also be tested in animals that may include guinea-pigs, adult mice, suckling mice and embryonated chicken eggs, as appropriate. For test details, refer to the Requirements for measles vaccines (live) (52). See also section A.4.2.1.1.

New molecular methods with broad detection capabilities are being developed for the detection of adventitious agents. These methods include: degenerate NAT for whole virus families with analysis of the amplicons by hybridization, sequencing or mass spectrometry; NAT with random primers followed by analysis of the amplicons on large oligonucleotide microarrays of conserved viral sequencing or digital subtraction of expressed sequences; and high-throughput sequencing. These methods may be used in the future to supplement existing methods, or as alternative methods to both in vivo and in vitro tests, after appropriate validation and approval by the NRA (51).

Each virus master and working seed lot should be tested for, and shown to be free from, *Mycobacterium avium*, by an appropriate test approved by the NRA.

NAT may be used as an alternative to the mycobacteria microbiological culture method and/or to the in vivo guinea-pig test for the detection of mycobacteria, after suitable validation and approval by the NRA (51).

Additional testing for ALVs and adventitious agents may be performed on control eggs for the virus working seed lot (e.g. fowl pox, salmonella, mycobacteria).

A.4.2.5 Tests in nonhuman primates

Each virus master and working seed lot should be tested for neurotropism, viscerotropism and immunogenicity in nonhuman primates, as described in Appendix 2.
A.4.2.6  **Virus titration for infectivity**

Each virus master and working seed lot should be assayed for yellow fever virus infectivity in a sensitive assay in cell cultures, as described in Appendix 4.

A.5  **Control of vaccine production**

Penicillin and other beta-lactams should not be used at any stage of the manufacture because of their nature as highly sensitizing substances. Other antibiotics may be used if approved by the NRA, and provided that the quantity present in the final product is acceptable to the NRA.

A.5.1  **Tests on uninoculated control eggs**

If monitoring of the flocks supplying embryonated chicken eggs is not under the direct responsibility of the vaccine manufacturer, an SPF certificate and quality control certificate (with test results) should be available from the supplier. The test described next should be performed.

A sample of 2% (which should comprise at least 20 but no more than 80 eggs) of the uninoculated embryonated eggs from the batch used for vaccine production should be incubated under the same conditions as the inoculated embryonated eggs. At the time of virus harvest, the uninoculated embryonated eggs should be processed in the same manner as the inoculated embryonated eggs, and the extract from the control embryos must be shown to be free from haemagglutinating agents, ALVs and other adventitious agents, by methods approved by the NRA.

A.5.2  **Single harvests**

After inoculation and incubation at controlled temperature and humidity, living and normal chicken embryos only should be harvested. The age of embryos at the time of harvest should be calculated from the initial introduction of the eggs into the incubator and should be no more than 12 days. The number of rejected eggs may be estimated, to monitor the consistency of the production.

After homogenization and centrifugation, the embryonic extract should be kept at –60 °C or below until further processing.

All intermediates should be maintained under conditions shown by the manufacturer to retain the desired biological activity. Storage periods should be approved by the NRA.

A.5.3  **Tests on single harvests**

A.5.3.1  **Sampling**

Samples required for the testing of single harvests should be taken immediately on harvesting and prior to further processing. If the tests are not performed
immediately, the samples taken for tests on single harvests should be kept at a temperature of \(-60^\circ C\) or below and subjected to no more than one freeze/thaw cycle.

### A.5.3.2 Identity

Each single harvest or group of single harvests from a daily production should be identified as yellow fever virus by immunological assay, on cell culture using specific antibodies or by molecular methods approved by the NRA.

### A.5.3.3 Tests for bacteria, fungi and mycoplasmas

Each single harvest or group of single harvests from a daily production should be tested for bacterial, fungal and mycoplasmal contamination by appropriate tests, as specified in Part A, sections 5.2 (49) and 5.3 (50) of General requirements for the sterility of biological substances, or by a method approved by the NRA.

NAT alone or in combination with cell culture, with an appropriate detection method, may be used as an alternative to one or both of the compendial mycoplasma detection methods, after suitable validation and agreement by the NRA (51).

### A.5.3.4 Tests for adventitious agents

Each single harvest or group of single harvests from a daily production should be tested for and shown to be free from *Mycobacterium avium* by an appropriate test approved by the NRA.

NAT may be used as an alternative to mycobacteria microbiological culture method and/or the in vivo guinea-pig test for the detection of mycobacteria, after suitable validation and approval by the NRA (51).

### A.5.3.5 Virus titration

The live yellow fever virus content of each single harvest or group of single harvests from a daily production should be determined by titration in cell culture against a reference preparation and the titre should be expressed in IU/ml (see Appendix 4).

### A.5.4 Final bulk

The final bulk should be prepared from one or several single harvests. The addition of any stabilizing agents should be approved by the NRA. The tests described next should be performed unless they have already been performed on each single harvest. The final bulk should, in any case, be tested for sterility. Samples that are
not tested immediately should be stored at or below –60 °C and subjected to no more than one freeze/thaw cycle.

A.5.4.1 Sterility tests for bacteria and fungi
Each final bulk should be tested for bacterial and fungal sterility as specified in Part A, section 5.2 of General requirements for the sterility of biological substances (49), or by the methods approved by the NRA.

A.5.4.2 Stabilizers
If a stabilizing agent is added, its concentration should be measured. The method used and permitted levels should be approved by the NRA.

A.5.4.3 Virus titration (if performed)
The live yellow fever virus content of each final bulk should be determined by titration in cell culture against a reference preparation, and the titre should be expressed in IU/ml (see Appendix 4).

A.6 Filling and containers
The general requirements concerning filling and containers given in Good manufacturing practices for biological products (48) should apply to yellow fever vaccine. Care must be taken to ensure that the materials of which the container, and if applicable the closure, are made do not adversely affect the virus content of the vaccine under the recommended conditions of storage. The vaccine should be freeze-dried.

Single- and multiple-dose containers may be used.

Failure to achieve adequate drying will result in a product that is susceptible to rapid deterioration even at 0 °C. Since yellow fever virus is extremely labile, unless the container is well sealed, variations in virus content may occur during storage. The manufacturer should ensure that the seal is satisfactory.

The manufacturer should provide the NRA with adequate data to prove the stability of the vaccine under appropriate conditions of storage and shipping (see section A.12).

A.7 Control tests on final lot
Samples should be taken from each final vaccine lot for testing and should fulfil the requirements of this section. All the tests and specifications, including the
methods used and the permissible limits for the different parameters listed under this section, unless otherwise specified, should be approved by the NRA.

A.7.1 **Inspection of final containers**

Every container in each final lot should be inspected visually, and those showing abnormalities should be discarded.

A7.1.1 **Appearance**

The appearance of the freeze-dried vaccine and the reconstituted vaccine should be described with respect to their form and colour. If reconstitution with the product diluent does not allow for the detection of particulates, an alternative diluent may be used.

If the glass used for the final containers does not permit inspection of the contents (e.g. with tinted glass), visual inspection should be performed on the reconstituted vaccine and the observations should comply with the specifications approved by the NRA.

A.7.2 **Identity**

An identity test should be performed on at least one container from each final lot after reconstitution of the vaccine according to the indications of the manufacturer for preparing the vaccine for human administration. A high-titre, monospecific immune serum or a monoclonal antibody known to be free from neutralizing agents that react with other flaviviruses should be used.

A sensitive test in cell cultures (plaque-reduction test) should be used for the identity test. Dilutions of vaccine are mixed with immune and non-immune serum. A suitable immunogenicity test is described in Appendix 2. If a 50% reduction in plaque number at the 1:10 dilution is not observed for the vaccine mixed with immune serum compared with vaccine mixed with non-immune serum, the vaccine should be rejected.

Molecular tests may also be used after suitable validation and approval by the NRA.

A.7.3 **Potency**

Three final containers should be selected at random from each final lot and should be individually tested on the same day against a reference preparation of yellow fever vaccine calibrated in IU and approved by the NRA. The containers should be assayed in cell cultures demonstrated to be of adequate sensitivity and approved by the NRA (see Appendix 4).

Before assay but after reconstitution of the vaccine in the volume and diluent recommended by the manufacturer for preparation for human
administration, the vaccine should be held at a temperature between 20 °C and 30 °C for 20 minutes, before further dilution. This material should be considered as undiluted vaccine. 

The dose recommended for use in humans should not be less than 3.0 \log_{10} IU. The release specification should be approved by the NRA.

An internal upper limit may be established by each manufacturer to monitor the consistency of production (e.g. based on mean titre in IU/dose +3 standard deviations). The upper limit should be approved by the NRA.

Existing release specifications should not be changed unless justified by clinical data and approved by the NRA. Major changes to existing vaccines (e.g. during production or in formulation and which may have a potential impact on the efficacy of the vaccine) should be justified by clinical data and approved by the NRA.

Specifications for new manufacturers (including manufacturers with production transfer) should be set by clinical trial and expressed in IU.

A.7.4 Thermal stability

The thermostability test aims to demonstrate consistency of production. Additional guidance on the evaluation of vaccine stability is provided in WHO’s Guidelines for stability evaluation of vaccines (43).

Three final containers from the freeze-dried final lot should be incubated at 37 °C for 2 weeks. These containers should be titrated in parallel with three containers that have been stored at or below the recommended storage temperature. A reference preparation calibrated in IU and approved by the NRA should be included in each assay. At the end of the incubation period, the geometric mean infectious titre in the incubated final containers should not have decreased by more than 1.0 \log_{10} IU.

A.7.5 Sterility tests for bacteria and fungi

Each final lot should be tested for bacterial and fungal sterility as specified in Part A, section 5.2 of General requirements for the sterility of biological substances (49), or by the methods approved by the NRA.

A.7.6 General safety test

Each final lot should be tested for the absence of abnormal toxicity in mice and guinea-pigs, using a general safety (innocuity) test approved by the NRA, and should pass the test.

This test may be omitted for routine lot release once consistency of production has been established to the satisfaction of the NRA.
A.7.7  **Residual moisture**
The residual moisture in a representative sample of each freeze-dried final lot should be determined by a method approved by the NRA. The upper limit of the moisture content should be approved by the NRA on the basis of stability tests.

A.7.8  **Residual ovalbumin**
The content of residual ovalbumin should be determined and should be within limits approved by the NRA.

A.7.9  **Endotoxin content**
The vaccine in the final container should be tested for endotoxin with a *Limulus* amoebocyte lysate test. The endotoxin content should be consistent with levels found to be acceptable in vaccine lots used in clinical trials and approved by the NRA.

A.7.10  **Residual antibiotics (if applicable)**
If any antibiotics are added during vaccine production, the content of the residual antibiotics should be determined and must be within limits approved by the NRA.

A.8  **Records**
The requirements given in section 8 of Good manufacturing practices for biological products (48) should apply.

A.9  **Retained samples**
The requirements given in section 9.5 of Good manufacturing practices for biological products (48) should apply.

A.10  **Labelling**
The requirements given in section 7 of Good manufacturing practices for biological products (48) should apply, with the additions listed next.
The label on the carton or the leaflet accompanying the container should:

- state that the vaccine fulfils Part A of these Recommendations;
- state the nature of the preparation, specify the substrain of yellow fever virus in the vaccine and the minimum number of infectious units per human dose, and state that SPF eggs were used;
- state the nature and quantity of any residual antibiotic present in the vaccine;
- indicate that the vaccine contains proteins derived from eggs;
indicate that contact of the vaccine with disinfectants is to be avoided;
indicate that the dose should be the same for persons of all ages;
indicate the volume and nature of the diluent to be added to reconstitute the vaccine, and specify that only the diluent supplied by the manufacturer should be used;
state that the vaccine is contraindicated in children aged under 6 months and is not recommended for those aged 6–8 months (17), except in specific circumstances, and it should be in accordance with available official recommendations;
state that the reconstituted vaccine should be used without delay or, if not used immediately, that it should be stored between 2 °C and 8 °C, protected from direct light and used within six hours (53).

A.11 Distribution and shipping
The requirements given in section 8 of Good manufacturing practices for biological products (48) should apply. Further guidance is provided in WHO's Model guidance for the storage and transport of time- and temperature-sensitive pharmaceutical products (54).

A.12 Stability, storage and expiry date
A.12.1 Stability testing
Adequate stability studies form an essential part of vaccine development. Current guidance on the evaluation of vaccine stability is provided in WHO's Guidelines for stability evaluation of vaccines (43). Stability testing should be performed at different stages of production, namely on single harvests or pools of single harvests, final bulk and final lot. In addition, such studies should be undertaken on reconstituted vaccine. Stability-indicating parameters should be defined or selected appropriately, according to the stage of production. It is advisable to assign a shelf-life to all in-process materials during vaccine production – particularly intermediate materials such as single harvests and final bulk.

The stability of the vaccine in its final container and at the recommended storage temperatures should be demonstrated to the satisfaction of the NRA on at least three consecutive lots of final product. Accelerated thermal stability tests may be undertaken to give additional information on the overall stability of a vaccine.

The formulation of vaccine should be stable throughout its shelf-life. Acceptable limits for stability should be agreed with the NRA. Following licensure, ongoing monitoring of vaccine stability is recommended, in order to support shelf-life specifications and refine the stability profile (43). Data should be provided to the NRA, in accordance with regulatory requirements.
A.12.2 Storage conditions
Before being distributed by the manufacturing establishment, or before being issued from a depot for the maintenance of vaccine reserves, all vaccines should be kept, at all times, at a temperature approved by the NRA.

The manufacturer should recommend conditions of storage and shipping that will ensure that the vaccine conforms to the requirements of potency until the expiry date stated on the label. These conditions should be approved by the NRA. The vaccine should have been shown to meet the release specifications for a period equal to that between the date of release and the expiry date.

A.12.3 Expiry date
The expiry date should be defined on the basis of shelf-life and should be supported by the stability studies with the approval of the NRA.

A.12.4 Expiry of reconstituted vaccine
For single-dose containers, the reconstituted vaccine should be used immediately. For multidose containers, the container should be kept in the dark at 2–8 °C. The expiry time for use of an opened container should be defined by stability studies and approved by the NRA, but should not be more than six hours (54).

Part B. Nonclinical evaluation of live attenuated yellow fever vaccines
The nonclinical evaluation of candidate live attenuated yellow fever vaccines derived from substrains of the 17D strain should be based on the WHO guidelines on nonclinical evaluation of vaccines (12).

Any new candidate 17D strain that is not already in use by a manufacturer should be characterized with respect to immunogenicity and safety and should be compared to at least one strain in current use for the manufacture of a licensed vaccine. In the case of manufacturing changes for an existing vaccine, recharacterization of the vaccine strain may be required.

The specific issues discussed next should be considered.

B.1 Characterization of a new candidate yellow fever vaccine
Any new candidate virus used in the production of vaccine requires supporting data that would qualify it for use. The new candidate virus should be identified by historical records that include information on the origin of the virus, its method of attenuation, whether the virus has been biologically or genetically cloned prior to generation of the master seed, genetic sequence information and the passage level.
To assess genotypic and phenotypic stability, virus from each production passage level should be characterized by laboratory and animal tests, in comparison with a currently acceptable vaccine. These tests may include full genome sequencing, growth in permissive and semi-permissive cell cultures, plaque-size estimation by plaque assays, and mosquito infectivity and dissemination.

Seed viruses used in the manufacture of vaccine intended for clinical trials should be tested as described in Appendix 2, to demonstrate that the seed virus is suitable for use in vaccine production.

B.2 Immunogenicity and other pharmacodynamic studies
The nonclinical studies should indicate that the new candidate yellow fever vaccine induces neutralizing antibodies to yellow fever virus in mice and nonhuman primates. A currently licensed yellow fever vaccine should be included as a control in such studies.

B.3 Toxicity assessment
In the early development of a new candidate yellow fever vaccine, and prior to the initiation of clinical trials in humans, toxicity assessment – including systemic toxicity and local tolerance – should be considered in relevant species in accordance with WHO guidelines (12). The toxicology assessment should include an evaluation of neurotropism and viscerotropism. If the candidate vaccine is to be licensed to include women of childbearing potential, a reproductive toxicity study will need to be conducted at an appropriate point in development, in accordance with WHO guidelines (12), and would require administration of the vaccine to pregnant animals in the early phase of implantation/organogenesis, since this is the phase that is most at risk.

These studies must demonstrate that the new candidate yellow fever vaccine is safe and suitable for use in humans.

Appropriate safety characterization studies should be conducted, and should include an evaluation of neurotropism and viscerotropism, according to the accepted protocol, which suggests monkey as the relevant species and the use of the 17D vaccine as a comparator (see section A.4.2.2.5 and Appendix 2).

Part C. Clinical evaluation of live attenuated yellow fever vaccines
Clinical trials should adhere to the principles described in the WHO Guidelines for good clinical practice (GCP) for trials on pharmaceutical products (55) and to the WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (13). All clinical trials should be approved by the relevant NRAs.
Some of the issues that are specific to the clinical evaluation of yellow fever vaccines derived from the 17D strain are discussed in the following sections. These sections should be read in conjunction with the general guidance mentioned above. It is also recommended that manufacturers should consult with relevant NRAs regarding the overall clinical development programme.

Part C considers the provision of clinical data required (i) when a new candidate live attenuated yellow fever vaccine derived from the 17D virus is developed; and (ii) when there have been major changes to the manufacturing process of an established vaccine (including preparation of a new virus master seed lot of an established strain). Clinical evaluation of vaccine manufactured using a new working seed lot is not required, provided that the passage level is not more than one from the master seed lot, the working seed has been characterized, and consistency of the manufacturing process has been demonstrated.

C.1 General considerations
Due to the success of 17D vaccines over the past 70 years, studies of vaccine efficacy are not feasible. Therefore, clinical studies should assess the safety and immunogenicity of a candidate yellow fever vaccine in comparison with at least one licensed vaccine. The assessment of immunogenicity should be based on the elicitation of neutralizing antibodies, which are thought to be the basis of protection (56), although the actual mechanism of protection is not known (57–59).

The relative risk of YEL-AVD and YEL-AND for a new candidate yellow fever vaccine versus approved vaccines cannot be estimated from pre-approval studies but should be addressed as part of post-marketing surveillance.

C.2 Safety and immunogenicity studies
C.2.1 Assessment of the immune response
The demonstration of an immune response to vaccination should be based on the measurement of neutralizing antibody titres both pre- and post-vaccination. Neutralizing antibody may be determined either by the plaque-reduction neutralization test (PRNT) or by using the log10 neutralization index (LNI). Geometric mean titres (GMTs), seroconversion rates and reverse cumulative distributions (RCDs) should be provided. Seroconversion may be defined as either a fourfold increase in neutralizing antibody or the induction of measurable neutralizing antibody in a previously seronegative individual. It is desirable to consider these two phenomena separately in the comparison between a novel 17D vaccine and a licensed one used as control.

The flavivirus haemagglutination inhibition (HAI) test may be used to determine whether or not individuals enrolled in vaccine studies are flavivirus naïve (see below). This test is not suitable for assessing responses to vaccination.
C.2.2  **Immunogenicity studies**

New candidate yellow fever vaccines (i.e. manufactured using a newly derived 17D strain) should be compared with at least one well-established and licensed 17D yellow fever vaccine. It is preferable that the comparative vaccine(s) selected should have been in widespread use for some years, so that some data on effectiveness are available as well as a reliable description of the safety profile.

If the candidate vaccine has been produced by an existing manufacturer from a new virus master seed lot, the comparison should be against a lot derived from the existing virus master seed.

C.2.3  **Population**

Safety and immunogenicity studies should be undertaken initially in healthy adults aged 18–60 years, preferably in need of vaccination against yellow fever. Subjects may be resident in non-endemic or endemic areas and should have no history of yellow fever or vaccination against yellow fever.

Studies in children should be undertaken only after adult studies have demonstrated that the safety profile is acceptable. In accordance with national and regional recommendations, it is likely that inclusion of children aged 9 months or more would be possible and desirable in endemic countries. However, some NRAs have agreed that studies in children are not always required, provided that the studies in adults are satisfactory and the overall experience with the use of 17D vaccines in children is taken into account.

The study exclusion criteria should reflect the current contraindications to administration of live attenuated yellow fever vaccines (e.g. pregnancy, known allergy to vaccine components, and immunosuppression).

C.2.4  **End-points and analyses**

The protocol should state the primary objective(s) of the study. The neutralizing antibody response to the candidate vaccine should be demonstrated to be non-inferior compared to an appropriate licensed yellow fever vaccine based primarily on GMTs and/or seroconversion rates. The primary end-point should be selected according to the study population and the anticipated immune response. For example, very high seroconversion rates are expected in healthy adults, and this has implications for the selection of the non-inferiority margin and thus the sample size calculation. Further details on demonstrating non-inferiority are described in WHO’s Guidelines on clinical evaluation of vaccines: regulatory expectations (13).

The primary analysis should be conducted in subjects who are flavivirus-naïve. If the HAI results are obtained only after vaccination (rather than being used to screen subjects for study eligibility before enrolment), the results for neutralizing antibody against yellow fever should be analysed both overall and
separately for those who were flavivirus-naive or non-naive, in order to assess any effect of pre-existing antibody to a heterologous flavivirus (e.g. dengue or West Nile viruses) on the response to yellow fever vaccine.

Other immunological parameters should be compared in planned secondary analyses (e.g. percentages reaching predefined titres).

C.2.5  **Dose-ranging studies**

Dose-ranging studies may be undertaken for new vaccines based on a 17D virus seed, to determine the minimum dose of virus (in IU) required to provide adequate immune responses. These data can also be used to support the derivation of the minimum viral titre that should be present in the vaccine at the end of its shelf-life. The assessment of safety of a 17D yellow fever vaccine during clinical studies should be in accordance with WHO’s Guidelines on clinical evaluation of vaccines: regulatory expectations (13).

C.2.6  **Concomitant administration with other vaccines**

An evaluation of the effects of co-administration of a yellow fever vaccine with other vaccines should be considered, taking into account which vaccines are most likely to be given concomitantly in different age groups and populations.

If a yellow fever vaccine is to be used in an EPI programme simultaneously with other vaccines, it is particularly important that the effects of co-administration should be evaluated. For example, some studies in children have shown that co-administration with measles–mumps–rubella (MMR) combined vaccines has resulted in lowered serological responses to yellow fever vaccines (60).

Immune responses to all other antigens co-administered with the yellow fever vaccine should be measured at least in subsets. While the study will usually be powered only to demonstrate non-inferiority with respect to neutralizing antibody against yellow fever, the protocols should at least include planned secondary analyses of antigen-specific responses. If these analyses indicate that immune responses are lower on co-administration with a new yellow fever vaccine compared to the licensed vaccine(s), NRAs will need to consider the potential clinical consequences on a case-by-case basis.

C.2.7  **Viraemia**

Assessment of viraemia is not routinely required for a 17D-derived vaccine because it is usual that recipients of yellow fever vaccines have a transient viraemia.

A low-level viraemia is known to occur after 17D vaccination. Titres of virus in blood have traditionally been determined by counting plaques in tissue culture monolayers that have been infected with serial dilutions of serum samples. More recently, reverse transcriptase polymerase chain reaction (RT-PCR) and quantitative RT-PCR have been used instead of the plaque assay. Quantitative
RT-PCR assays should include the generation of a standard curve using quantitative RT-PCR of 17D vaccine virus, so that the results can be expressed as plaque-forming unit (PFU) equivalents or genomic equivalents. There is currently no International Standard available for quantitative RT-PCR assays.

C.2.8 Pre-licensure safety data
The general approach to the assessment of safety of a new 17D yellow fever vaccine during clinical studies should be in accordance with WHO’s Guidelines on clinical evaluation of vaccines: regulatory expectations (13). Planned safety studies should be supported by a clear scientific rationale. However, given the long history of the use of 17D vaccines, the NRA may decide that sufficient data can be obtained from the immunogenicity studies in relatively small numbers. Where a new 17D seed that has not been used previously is investigated, larger-scale studies may be needed.

An appropriate pharmacovigilance plan should be developed and approved by the NRA prior to licensure.

C.3 Post-marketing studies and surveillance
Enhanced safety surveillance (particularly for the detection of YEL-AND and YEL-AVD) should be undertaken during the initial post-approval years, in collaboration with NRAs. The total duration of enhanced surveillance should be regularly reviewed by the NRA. Case definitions for YEL-AVD are being developed by the Brighton Collaboration and should be used when they are finalized (61, 62).

If particular issues arise during pre-licensure studies or during post-licensure safety surveillance, it may be necessary to conduct specific post-licensure safety studies.

Part D. Recommendations for national regulatory authorities

D.1 General
The general recommendations for control laboratories given in the Guidelines for national authorities on quality assurance for biological products (63) and Guidelines for independent lot release of vaccines by regulatory authorities (64) should apply. These Guidelines specify that no new biological substance should be released until consistency of manufacturing and quality, as demonstrated by a consistent release of batches, has been established. The detailed production and control procedures, and any significant changes in them that may affect the quality, safety and efficacy of yellow fever vaccine, should be discussed with,
and approved by, the NRA. For control purposes, the NRA should obtain the International Standard for potency testing and, where necessary, should establish national working reference preparation(s) calibrated against the International Standard.

D.2 Release and certification

A vaccine lot should be released only if it fulfils the national requirements and/or Part A of the current Recommendations. A protocol based on the model given in Appendix 5, and signed by the responsible official of the manufacturing establishment, should be prepared and submitted to the NRA in support of a request for release of a vaccine for use. A statement signed by the appropriate official of the NRA should be provided, if requested by a manufacturing establishment, and should certify whether or not the lot of vaccine in question meets all national requirements, as well as Part A of these Recommendations. The certificate should also state the lot number, the number under which the lot was released, and the number appearing on the labels of the containers. In addition, the date of the last satisfactory potency test, as well as the assigned expiry date on the basis of shelf-life, should be stated. A copy of the official national release document should be attached. The certificate should be based on the model given in Appendix 6. The purpose of the certificate is to facilitate the exchange of vaccines between countries.

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Dr D. Akemi Hokama, Bio-Manguinhos, Rio de Janeiro, Brazil; Dr A. Bentsi-Enchill, World Health Organization, Geneva, Switzerland; Dr N. Boschetti, Crucell, Berna Ltd, Thörishaus, Switzerland; Mr A. Costa, World Health Organization, Geneva, Switzerland; Dr P. Cottin, Sanofi Pasteur, Lyons, France; Dr A. Diatta, Institut Pasteur de Dakar, Dakar, Senegal; Dr G. Dong, National Institute for the Control of Pharmaceutical & Biological Products, Beijing, China; Dr K. Eckels, Walter Reed Army Institute of Research, Silver
Spring, MD, USA; Ms F. Garnier, Agence Française de Sécurité Sanitaire des
Produits de Santé, Lyons, France; Dr L. Gerentes, Sanofi Pasteur, Val de Reuil
Cedex, France; Dr G. Girault, Institut Pasteur de Dakar, Dakar, Senegal; Dr P.
Gonnet, Sanofi Pasteur, Val de Reuil Cedex, France; Dr V. Grachev, Chumakov
Institute of Poliomyelitis and Viral Encephalitides of the Russian Academy of
Medical Sciences, Moscow, Russian Federation; Dr A. Kiktenko, Chumakov
Institute of Poliomyelitis and Viral Encephalitides of the Russian Academy of
Medical Sciences, Moscow, Russian Federation; Dr I. Knezevic, World Health
Organization, Geneva, Switzerland; Dr H. Langar, WHO Regional Office for the
Eastern Mediterranean, Cairo, Egypt; Dr R. Lewis, World Health Organization,
Geneva, Switzerland; Dr L. Markoff, Center for Biologics Evaluation and
Research, United States Food and Drug Administration, Rockville, MD, USA;
Dr R. Martins, Bio-Manguinhos, Rio de Janeiro, Brazil; Dr S. Morgeaux, Agence
Française de Sécurité Sanitaire des Produits de Santé, Lyons, France; Dr G.
Poumerol, World Health Organization, Geneva, Switzerland; Dr M. Powell,
Medicines and Healthcare Products Regulatory Agency, London, England; Dr A.
Sinyugina, Chumakov Institute of Poliomyelitis and Viral Encephalitides of the
Russian Academy of Medical Sciences, Moscow, Russian Federation; Dr J.
Stalder, Swissmedic, Berne, Switzerland; Dr R. Teyssou, Sanofi Pasteur, Lyons,
France; Dr M. Vorobieva, Tarasevich State Research Institute for Standardization
& Control of Medical Biological Preparations, Moscow, Russian Federation;
Dr D.J. Wood, World Health Organization, Geneva, Switzerland; Dr B. Zeng,
China National Biotec Group, Beijing, China.

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with support from the WHO secretariat: Dr T.Q. Zhou, Dr J-H. Shin, Dr I.
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into account comments received from:

Dr A. Barrett, University of Texas Medical Branch, Galveston, TX, USA;
Dr A. Bentsi-Enchill, World Health Organization, Geneva, Switzerland; Dr N.
Boschetti, Crucell, Berna Ltd, Thörishaus, Switzerland; Dr P. Cottin, Sanofi
Pasteur, Lyons, France; Dr A. Diatta, Institut Pasteur de Dakar, Dakar, Senegal;
Dr G. Dong, National Institute for the Control of Pharmaceutical & Biological
Products, Beijing, China; Dr K. Eckels, Walter Reed Army Institute of Research,
Silver Spring, MD, USA; Ms F. Garnier, Agence Française de Sécurité Sanitaire
des Produits de Santé, Lyons, France; Dr T. Gastineau, Sanofi Pasteur, Lyons,
France; Dr L. Gerentes, Sanofi Pasteur, Val de Reuil Cedex, France; Dr P.
Gonnet, Sanofi Pasteur, Val de Reuil Cedex, France; Dr S. Gould, Sanofi Pasteur,
Lyons, France; Dr V. Grachev, Chumakov Institute of Poliomyelitis and Viral
Encephalitides of the Russian Academy of Medical Sciences, Moscow, Russian
Federation; Dr A. Kiktenko, Chumakov Institute of Poliomyelitis and Viral
Encephalitides of the Russian Academy of Medical Sciences, Moscow, Russian
Federation; Dr W. Lapps, Sanofi Pasteur, Swiftwater, PA, USA; Dr M. da Luz
Fernandes Leal, Bio-Manguinhos, Rio de Janeiro, Brazil; Dr L. Mallet, Sanofi Pasteur, Lyons, France; Dr A. Malkin, Chumakov Institute of Poliomyelitis and Viral Encephalitides of the Russian Academy of Medical Sciences, Moscow, Russian Federation; Dr R. Martins, Bio-Manguinhos, Rio de Janeiro, Brazil; Dr P. Minor, National Institute of Biological Standards and Control, Potters Bar, England; Dr S. Morgeaux, Agence Francaise de Sécurité Sanitaire des Produits de Santé, Lyons, France; Dr A. Sinyugina, Chumakov Institute of Poliomyelitis and Viral Encephalitides of the Russian Academy of Medical Sciences, Moscow, Russian Federation; Dr J. Stalder, Swissmedic, Berne, Switzerland; Dr R. Teyssou, Sanofi Pasteur, Lyons, France; Dr J-W. van der Laan, Centre for Biological Medicines and Medical Technology, National Institute for Public Health and the Environment, Bilthoven, the Netherlands. Dr L.M. Correa Werneck, Oswaldo Cruz Foundation, National Institute of Quality Control in Health, Rio de Janeiro, Brazil; Dr C. Xu, China National Biotec Group, Beijing Tiantan Biological Products Co. Ltd, Beijing, China.

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Dr C.P. Alfonso, World Health Organization, Geneva, Switzerland; Dr M. Baca-Estrada, World Health Organization, Geneva, Switzerland; Dr A. Barrett, University of Texas Medical Branch, Galveston, TX, USA; Dr A. Bentsi-Enchill, World Health Organization, Geneva, Switzerland; Dr C.S-B. Boye, Institut des Sciences du Médicament, Université Cheikh Anta Diop, Dakar, Senegal; Dr E. Charton, European Directorate for the Quality of Medicines and HealthCare, Strasbourg, France; Dr C. Conrad, World Health Organization, Geneva, Switzerland; Dr A.M. Diatta, Institut Pasteur de Dakar, Dakar, Senegal; Dr G. Dong, National Institute for the Control of Pharmaceutical & Biological Products, Beijing, China; Dr K. Eckels, Walter Reed Army Institute of Research, Silver Spring, MD, USA; Dr M. Ferguson, Consultant, Horning, Norfolk, England; Mr M.F. Galves da Silva, Biological Products – ANVISA, Brasília, Brazil; Ms F. Garnier, Agence Française de Sécurité Sanitaire des Produits de Santé, Lyons, France; Dr V. Grachev, Chumakov Institute of Poliomyelitis and Viral Encephalitides of the Russian Academy of Medical Sciences, Moscow, Russian Federation; Dr C. Herzog, Crucell Switzerland Ltd., Bern, Switzerland; Dr J. Hombach, World Health Organization, Geneva, Switzerland; Dr A. Kiktenko, Chumakov Institute of Poliomyelitis and Viral Encephalitides of the Russian Academy of Medical Sciences, Moscow, Russian Federation; Dr I. Knezevic, World Health Organization, Geneva, Switzerland; Dr L. Mallet, Sanofi Pasteur, Toronto, Ontario, Canada; Dr L. Markoff, Center for Biologics Evaluation and
Research, United States Food and Drug Administration, Rockville, MD, USA; Dr R.M. Martins, Bio-Manguinhos, Rio de Janeiro, Brazil; Dr P. Minor, National Institute for Biological Standards and Control, Potters Bar, England; Dr S.A. Nishioka, World Health Organization, Geneva, Switzerland; Dr M. Powell, Medicines and Healthcare Products Regulatory Agency, London, England; Dr J. Shin, World Health Organization, Geneva, Switzerland; Dr A. Sinyugina, Chumakov Institute of Poliomyelitis and Viral Encephalitides of the Russian Academy of Medical Sciences, Moscow, Russian Federation; Dr J. Stalder, Swissmedic, Berne, Switzerland; Dr R. Teyssou, Sanofi Pasteur, Lyons, France; Dr D. Trent, Xcellerex, Marlborough, MA, USA; Dr J.W. van der Laan, Centre for Biological Medicines and Medical Technology, National Institute for Public Health and the Environment, Bilthoven, the Netherlands; Dr M. Vorobyeva, Tarasevich State Research Institute for Standardization & Control of Medical Biological Preparations, Moscow, Russian Federation; Dr L.M. Werneck, Oswaldo Cruz Foundation, National Institute of Quality Control in Health, Manguinhos, Brazil; Dr D.J. Wood, World Health Organization, Geneva, Switzerland; Dr C. Xu, Sionpharm-Beijing Tiantan Biological Products Co. Ltd, Beijing, China; Dr S. Yactayo, World Health Organization, Geneva, Switzerland; Dr T.Q. Zhou, World Health Organization, Geneva, Switzerland.

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References


Appendix 1

Genealogy of 17D yellow fever vaccine strains

Two live attenuated yellow fever vaccines were developed in the 1930s: the French neurotropic vaccine (FNV) prepared from wild-type strain French viscerotropic virus passaged in mouse brain, and the 17D vaccine, which was prepared from wild-type strain Asibi virus passaged in embryonated chicken eggs. Today, the 17D vaccine is the only type of yellow fever vaccine produced, since the use of FNV was found to be associated with a high incidence of encephalitic reactions in children (1).

The 17D vaccine was developed by Theiler and Smith in 1937 and has been shown to protect against all seven known genotypes of wild-type yellow fever virus.

Two substrains of the 17D vaccine are used in vaccine production today (see Figure 1) (2), namely 17D-204 and 17DD. Some vaccines are also prepared from a distinct substrain of 17D-204 (17D-213) using seed viruses 112/95 and 213/77.

The 17D-204 vaccine substrain is utilized in all countries, except Brazil, where the 17DD vaccine substrain is used. The 17D-204 vaccine was developed from the original attenuated 17D by continued chick embryo passage (without neuronal tissue) from passage 176 to passage 204. Subsequently, the virus was passaged in embryonated chicken eggs, and most currently manufactured vaccines are manufactured at passage levels between 235 and 240. The 17DD vaccine was derived by passage in whole-chick embryonic tissue with the neuronal tissue removed from passage 176–195; however, subsequent passages were undertaken independently in Brazil, such that its development differed from 17D-204. This vaccine was passaged in embryonated chicken eggs and all currently manufactured vaccines are at passage level 287. During the 1970s and 1980s it became apparent that some vaccines had been prepared in eggs contaminated with ALV and therefore a number of manufacturers prepared ALV-free seeds of 17D virus, in order to remove the endogenous retrovirus. The Robert Koch Institute in Germany, on behalf of WHO, established a new seed lot from the 17D-204 strain at passage 237, termed 213-77. This was certified free of ALV contamination and is used at passage 239–240 in embryonated chicken eggs (2, 3). 213-77 is considered by some (though not all) scientists as a substrain of 17D, owing to acquisition of an envelope protein glycosylation site compared to the 17D-204 substrain, and is sometimes referred to as 17D-213 (3).

Over the years, there have been many manufacturers of yellow fever vaccines (see Figure 1). The 17D-204 substrain vaccine has been manufactured
in France, Germany, India, the Netherlands, Senegal, South Africa, the United Kingdom and the USA, while the 17DD substrain vaccine has been manufactured in Brazil and Colombia. The 17D-213 substrain vaccine has been manufactured in Nigeria and the Russian Federation, and Berna Biotech (now Crucell) in Switzerland has developed a vaccine derived from seed virus 112/95, though it had not yet been marketed by Crucell at the time this report was prepared. At present, there are only six producers: Sanofi Pasteur in France and the USA (17D-204); Institut Pasteur in Dakar, Senegal (17D-204); the Chumakov Institute of Poliomyelitis and Viral Encephalitides of the Russian Academy of Medical Sciences, Russian Federation (17D-213); Beijing Tiantan Biological Products Co. Ltd, China (17D-204); and Bio-Manguinhos/FioCruz of Rio de Janeiro, Brazil (17DD). Currently, four manufacturers are prequalified by WHO to provide yellow fever vaccine for use in developing countries. The other manufacturers produce yellow fever vaccine for domestic use.

The 17D-204 vaccine virus genome is 10 862 nucleotides in length and encodes a 3411 amino acid polyprotein, which is flanked by a 5’ non-coding region of 118 nucleotides and a 3’ non-coding region of 511 nucleotides (4, 5). The 5’ terminus has a type 1 cap followed by two conserved nucleotides (AG) and the 3’ terminus lacks a poly A tract (4). The polyprotein encodes 10 proteins: the structural proteins capsid (C), membrane (M) and envelope (E) proteins are encoded by the N-terminal one third of the polyprotein; and the non-structural (NS) proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 are encoded by the C-terminal two thirds of the polyprotein. The major immunogen is the E protein, which encodes epitopes, inducing neutralizing antibodies that are primarily responsible for the protective immune response. Monoclonal antibodies have identified a number of overlapping epitopes on the E protein (6, 7). Physically, these epitopes are either yellow fever strain-specific, yellow fever type-specific, complex-specific or flavivirus genus common, while biologically some of the epitopes are associated with haemagglutination inhibition (HI), which may or may not be associated with neutralization (6–17). Overall, few epitopes are involved in neutralization and very few elicit high-titre neutralization (6, 12, 16). Monoclonal antibodies have been generated against yellow fever wild-type and vaccine strains. Wild-type-specific (6, 8, 10, 11, 14, 15, 17) and vaccine-specific epitopes (i.e. recognizing 17D and FNV viruses only) (7, 8, 10, 11, 14, 15, 17), and 17D-204 and 17DD substrain-specific epitopes (8, 9, 13, 14) have all been identified on the E protein. To date, few epitopes have been mapped to specific amino acids on the E protein: two yellow fever type-specific epitopes have been mapped to amino acids 71/72 and 153/155, a wild-type epitope to amino acid 173, and a 17D-204 substrain-specific epitope to amino acids 305 and 325 (18–20). Human cytotoxic T cell epitopes are found on the E structural protein and the NS1, NS2B and NS3 non-structural proteins (21–22).
Figure 1

History and genealogy of 17D vaccines and reference viruses: status as of October 2010

Passage level

<table>
<thead>
<tr>
<th>Passage</th>
<th>Vaccine Name</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>Asibi</td>
</tr>
<tr>
<td>176</td>
<td>17D</td>
</tr>
<tr>
<td>195</td>
<td>17DD Low</td>
</tr>
<tr>
<td>204</td>
<td>Columbia 88</td>
</tr>
<tr>
<td>228</td>
<td>RF 145-3</td>
</tr>
<tr>
<td>230</td>
<td>RML YF-1</td>
</tr>
<tr>
<td>231</td>
<td>RF Lot 1028</td>
</tr>
<tr>
<td>232</td>
<td>RF 505</td>
</tr>
<tr>
<td>233</td>
<td>RF 555</td>
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</tbody>
</table>

**France**

- S1
- S2

**USA**

- S2 (LF)
- V(LF)

**Australia**

- AB 616

**Netherlands**

- AB 617
- S1
- AB 617
- S1

**Senegal**

- IP/F1: 73-23
- IP/F2: 771-2

**Senegal (LF)**

- [Stamaril®]

**China**

- 600103 (1952) (**39**)
- 6613 (1966) (**48**)

**Brazil**

- Lot 774 (1942) (**40**)
- P3 (1962) (**41**)

**Other countries**

- S1
- S2

Legends:

- Passages contaminated by avian leukaosis virus
- Passages free from avian leukaosis virus
- 17D
- 17D-204
- 17DD Low
- RF
- RML
- RF Lot
- YF
- WHO
- OIE
- S2
- S1
- Working seed
- Master seed
- Leukosis-free vaccines
- Reference virus
- Manufacturer's master seed
- Vaccine
- CRUCELL-YF Flavimun®
- RKI-YF RKI yellow fever vaccine
- **EP** Egg passage

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*Note: The diagram is complex and requires careful reading to understand the genealogy and passages of the 17D vaccines and reference viruses.*
Annex 5

Figure 1 continued

a This diagram provides information on a historical overview of the use of strains derived from the 17D yellow fever vaccine strain (as of October 2010). It does not indicate any WHO qualification or approval of the strains or vaccines in the context of this document.

The First International Standard for yellow fever vaccine (Code 99/616) was derived from a bulk vaccine derived from seed S2 YFS/10-11 (England).

The 17D-204 vaccines from Australia, Colombia, Germany, India, the Netherlands and South Africa, plus the 17DD vaccines from Colombia and Senegal, are not manufactured today.

Source: reproduced, with the permission of the publisher, from reference 2.

The genomes of 17DD (23, 24), 17D-204 (4, 5) and 17D-213 (23, 24) vaccine viruses and parent wild-type Asibi virus have been sequenced (25). Unfortunately, the original 17D virus is not available. The three substrains differ slightly in sequence, thus justifying their classification as substrains (24), but they share 20 amino acid substitutions and four nucleotide changes in the 3’ non-coding region. The capsid gene and 5’ non-coding region of wild-type Asibi and 17D vaccine viruses were identical in sequence (see Table 1). At present, the molecular basis of attenuation of 17D vaccine is not known. Mouse models indicate that multiple mutations may be responsible for the attenuated phenotype.

Genomic sequences have been published for various 17D vaccines, some by manufacturers and some by academic laboratories. These include vaccines prepared in Brazil (23, 24), China (unpublished Genbank accession # FJ654700), France (5, 26), Senegal (27), South Africa (28), USA (29) and the American Type Culture Collection (ATCC) (4). The original published sequence of 17D-204 vaccine (4) is based on the virus obtained from ATCC.

Table 1
Amino acid differences and nucleotide differences in the 3’ non-coding region between wild-type Asibi virus and attenuated 17D vaccines

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Gene</th>
<th>Amino acid&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Asibi</th>
<th>17D-204, 17D-213 and 17DD vaccine viruses</th>
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<tbody>
<tr>
<td>854</td>
<td>M</td>
<td>36</td>
<td>Leu</td>
<td>Phe</td>
</tr>
<tr>
<td>1127</td>
<td>E</td>
<td>52</td>
<td>Gly</td>
<td>Arg</td>
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<td>Thr</td>
<td>Ile</td>
</tr>
<tr>
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<td></td>
<td>200</td>
<td>Lys</td>
<td>Thr</td>
</tr>
<tr>
<td>1870</td>
<td></td>
<td>299</td>
<td>Met</td>
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Table 1 continued

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<th>Amino acid*</th>
<th>Asibi</th>
<th>17D-204, 17D-213 and 17DD vaccine viruses</th>
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</thead>
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<td></td>
<td>Phe</td>
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<td>6023</td>
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* The 20 amino acids and 4 nucleotide changes in the 3’ non-coding region identified in this table are conserved in any vaccine virus derived from the 17D strain.

References


Appendix 2

Tests in nonhuman primates of new virus master and working seeds

Each virus master and working seed lot should be tested for viscerotropism, immunogenicity and neurotropism, in a group of 10 test monkeys. Animals that are in the test vaccine and reference groups should be blinded to the operators throughout the experiment. For the neurotropism test, the test monkeys inoculated with the virus seed lot should be compared with a similar group of 10 monkeys injected with a reference virus.

A WHO reference virus, 168-73, is available from the National Institute for Biological Standards and Control, Potters Bar, England. This virus is of the same lineage as the WHO primary seed 213-77 (see Appendix 1, Figure 1), and unpublished data indicate that it is less neurovirulent in monkeys than strains of at least one other lineage known to produce an acceptable vaccine. Existing manufacturers should use a homologous reference; for instance, where their existing working seed is to be replaced by another derived from the same master seed, the existing seed can be used as the reference material, provided it has been shown to produce a vaccine with satisfactory properties. It is recommended that sufficient stocks of such a reference are kept for all future anticipated replacements of the working seeds.

It is likely, though unproven, that 168-73 will be a satisfactory reference for seeds of the 213-77 lineage.

A new manufacturer with a new seed should use a homologous preparation known to produce a satisfactory product as reference. The inclusion of 168-73 as a common material would make it possible to compare different tests and one lineage with another for information. The reference virus should be approved by the NRA.

The monkeys should be *Macaca mulatta* (i.e. rhesus monkeys) or *Macaca fascicularis* (i.e. cynomolgus monkeys) and should have been demonstrated to be non-immune to yellow fever virus by the haemagglutination inhibition test immediately prior to injection of the seed virus. They should be healthy and should not have been previously subjected to any experimentation. The test dose should be injected into one frontal lobe of each monkey, under anaesthetic, and the monkeys should be observed for a minimum of 30 days.

The test dose should consist of 0.25 ml containing not less than 5000 (3.7 log10) IU and not more than 50 000 (4.7 log10) IU, as shown by titration in
cell culture. In addition, the virus titre of the test virus seed lot and the reference virus should be as close as possible.

Historically, the test dose should consist of 0.25 ml containing the equivalent of not less than 5000 and not more than 50 000 mouse LD₅₀, as shown by a titration in cell culture.

1. Viscerotropism test

The criterion of viscerotropism (indicated by the amount of circulating virus) should be fulfilled as follows: sera obtained from each of the test monkeys on the second, fourth and sixth days after injection of the test dose should be inoculated at dilutions of 1:10, 1:100 and 1:1000 into at least four cell culture vessels per dilution. In no case should 0.03 ml of serum contain more than 500 (2.7 log₁₀) IU and in no more than one case should 0.03 ml of serum contain more than 100 (2.0 log₁₀) IU.

2. Immunogenicity test

The criterion of sufficient virus-neutralizing antibody in the sera (immunogenicity) should be fulfilled as follows: at least 90% of the test monkeys should be shown to have become immune within 30 days following injection of the test dose, as determined by examining their sera in the test for neutralization of yellow fever virus described below. In some countries it has been shown that, at low dilutions, some sera contain non-specific inhibitors that interfere with this test. The NRA may require sera to be treated to remove such substances.

Dilutions of 1:10, 1:40 and 1:160 of serum from each test monkey should be mixed with an equal volume of strain 17D vaccine virus at a dilution that has been shown to yield an optimum number of plaques when assayed according to one of the cell culture methods given in Appendix 4. These serum–virus mixtures should be incubated in a water bath at 37 °C for 1 h and then chilled in an ice-water bath before inoculation of 0.2 ml aliquots of each mixture into each of four separate cell culture vessels. The vessels should be handled according to one of the cell culture techniques described in Appendix 4. In addition, 10 vessels should be similarly inoculated with virus as above, and with an equal volume of a 1:10 dilution of monkey serum known to contain no neutralizing antibodies to yellow fever virus. At the end of the observation period, the mean number of plaques in the vessels receiving virus and non-immune serum should be compared with the mean number of plaques in the vessels receiving virus and serum from test monkeys. For the immunogenicity test to be satisfied, serum at the 1:10 dilution from no more than 10% of the test monkeys should fail to reduce the mean number of plaques by 50% as compared with the vessels containing non-immune serum.
3. Neurotropism test

Monkeys in the test group should be compared with 10 monkeys injected with the reference virus, with respect to both the clinical evidence of encephalitis and the severity of histological lesions of the nervous system (1, 2).

The onset and duration of the febrile reaction should not differ between monkeys injected with the test virus or with the reference virus.

3.1 Clinical evaluation

The monkeys should be examined daily for 30 days by personnel familiar with the clinical signs of encephalitis in primates.

If necessary, the monkeys may be removed from their cages and examined for signs of motor weakness or spasticity, as described elsewhere (2).

Signs of encephalitis – such as paresis, incoordination, lethargy, tremors or spasticity – should be assigned numerical values for severity by the following grading method. Each day each monkey should be given a numerical score based on the scale:

1: rough coat, not eating;
2: high-pitched voice, inactive, slow moving;
3: shaky movements, tremors, incoordination, limb weakness;
4: inability to stand, limb paralysis or death.

A monkey that dies receives the score “4” from the day of death until day 30.

The clinical score for a monkey is the average of its daily scores; the clinical score for a group is the arithmetic mean of the individual scores. For the clinical criterion of the neurotropism test to be satisfied, the clinical score of the monkeys injected with the virus being tested should not exceed the clinical score of the monkeys injected with the reference virus.

3.2 Histological evaluation

The cervical and lumbar enlargements of the spinal cord and specific structures at five levels of the brain should be examined (2) (see Appendix 3). The cervical and lumbar enlargements should each be divided equally into six blocks. The blocks should be dehydrated and embedded in paraffin wax; 15-µm sections should be cut and stained with gallocyanin. One section, consisting of two hemisections, should be cut from each block.

Tissue blocks 3–4 mm thick should be taken from the brain by making the following frontal cuts:
Block I:  the corpus striatum at the level of the optic chiasma;
Block II:  the thalamus at the level of the mamillary bodies;
Block III:  the mesencephalon at the level of the superior colliculi;
Block IV:  the pons and cerebellum at the level of the superior olives;
Block V:  the medulla oblongata at the midlevel of the inferior olives.

These blocks should be dehydrated and embedded in paraffin wax, and 15 µm sections should be cut and stained with gallocyanin. A single section, consisting of two hemisections, should be cut from each block.

Sections should be examined microscopically and numerical scores should be given to each hemisection of the lumbar and cervical cord enlargements and to each anatomical structure (see Appendix 3) within each hemisection of the brain blocks, according to the following grading system:

1 (minimal):  1–3 small, focal inflammatory infiltrates. A few neurons may be changed or lost;
2 (moderate):  more extensive focal inflammatory infiltrates (neuronal changes or loss affects no more than one third of neurons);
3 (severe):  neuronal changes or loss of 33–90% of neurons, with moderate focal or diffuse inflammatory infiltration;
4 (overwhelming):  more than 90% of neurons are changed or lost, with variable, but frequently severe, inflammatory infiltration.

Each brain block contains several anatomical structures, which contribute in different ways to the assessment of a test sample. For example, certain structures differentiate more reproducibly than others between acceptable and unacceptable yellow fever seed lots and vaccines (2). These are called discriminator areas, whereas structures that are more susceptible to yellow fever virus replication are called target areas. Though both rhesus and cynomolgus monkeys are acceptable, the discriminator and target areas are different for the two species. The major difference is that in cynomolgus monkeys the cervical and lumbar cord are target areas, whereas in rhesus monkeys they are discriminator areas. The footnotes to the worksheets (Appendix 3) indicate in more detail the discriminator and target areas for the two species. The worksheets also list other anatomical structures that will be present in the brain sections but that are not included in the evaluation of a test sample because they are rarely affected (spared areas).

Three separate scores should be calculated for each monkey: discriminator areas only, target areas only and discriminator plus target areas. These scores should be calculated as shown in the sample worksheets provided in Appendix 3.
Overall mean scores should also be calculated for each group of monkeys as the arithmetic mean of individual monkey scores for discriminator areas only and for discriminator plus target areas. Both overall mean scores should be considered when determining virus seed lot acceptability. For the histological criterion of the neurotropism test to be satisfied, both overall mean scores for the test monkeys should not be significantly greater (at the 5% significance level) than the overall mean scores for the monkeys injected with the reference virus.

Both the clinical and histological criteria of the neurotropism test should be satisfied, in order for the virus seed lot to meet the requirement for neurotropism.

References

Appendix 3

Example, for guidance, of a summary protocol for the testing of yellow fever vaccine in the monkey safety test as described in Appendix 2

Species: ________________________________
Number of monkeys inoculated: ________________________________
Master virus seed lot number: ________________________________
Reference virus lot number: ________________________________
Date of serology tests before inoculation: ________________________________
Dilution of yellow fever virus used for the inoculation: ________________________________
Volume and route of inoculation: ________________________________
Date of inoculation: ________________________________
Number of IU inoculated: ________________________________
Date of end of the test: ________________________________
Viscerotropism test (virus master seed lot)
Specify cell line used for virus titration.

<table>
<thead>
<tr>
<th>Monkey no.</th>
<th>Titre of circulating virus on:</th>
<th>Maximum titre of circulating virus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 2</td>
<td>Day 4</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
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<tr>
<td>3</td>
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<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Result (pass or fail) ____________________________________________________________
Immunogenicity test (virus master seed lot)
Specify cell line used for virus titration.

<table>
<thead>
<tr>
<th>Monkey no.</th>
<th>Seroneutralization titre</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
</tr>
<tr>
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</tr>
<tr>
<td>9</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

Result (pass or fail) ________________________________
**Neurotropism test (virus master seed lot)**

Summary clinical results

Date of inoculation: ________________

<table>
<thead>
<tr>
<th>Master virus seed lot no.</th>
<th>Clinical score</th>
<th>Reference virus lot no.</th>
<th>Clinical score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>2</td>
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<td></td>
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<td></td>
<td>15</td>
<td></td>
</tr>
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<td></td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>20</td>
<td></td>
</tr>
<tr>
<td><strong>Group mean</strong></td>
<td></td>
<td><strong>Group mean</strong></td>
<td></td>
</tr>
</tbody>
</table>

Result (pass or fail) ____________________________________________
**Histological worksheet**

The worksheets below are provided as examples of how the histological score is calculated for a cynomolgus monkey with lesions graded as shown.

Species: cynomolgus  
Pathology no: _______________  
Monkey no: _______________

<table>
<thead>
<tr>
<th>Corpus striatum and thalamus</th>
<th>Block I</th>
<th>Block II</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>N. caudatus&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1</td>
<td>0</td>
<td>2 (5/4 =) 1.25</td>
</tr>
<tr>
<td>Globus pallidus&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0</td>
<td>1</td>
<td>2 (3/4 =) 0.75</td>
</tr>
<tr>
<td>Putamen&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2</td>
<td>0</td>
<td>1 (4/4 =) 1.00</td>
</tr>
<tr>
<td>N. ant./med. thalamus&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1</td>
<td>1</td>
<td>0 (3/4 =) 0.75</td>
</tr>
<tr>
<td>N. lat. thalamus&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1</td>
<td>2</td>
<td>1 (5/4 =) 1.25</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>0</td>
<td>1</td>
<td>0 (1/4 =) 0.25</td>
</tr>
</tbody>
</table>

<sup>a</sup> Discriminator area for rhesus.  
<sup>b</sup> Discriminator area for cynomolgus.

<table>
<thead>
<tr>
<th>Mesencephalon (Block III)</th>
<th>L</th>
<th>R</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colliculi superior</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Corpus geniculatum med.</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N. oculomotorius</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N. ruber</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Substantia nigra&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2</td>
<td>2</td>
<td>(4/2 =) 2.00</td>
</tr>
</tbody>
</table>

<sup>c</sup> Target area for rhesus and cynomolgus.

<table>
<thead>
<tr>
<th>Pons (Block IV)</th>
<th>L</th>
<th>R</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>N. abducens</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N. vestibularis</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N. trigeminus</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N. facialis</td>
<td>0</td>
<td>1</td>
<td>(1/2 =) 0.5</td>
</tr>
</tbody>
</table>
Table continued

### Pons (Block IV)

<table>
<thead>
<tr>
<th></th>
<th>L</th>
<th>R</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formatio reticularis</td>
<td>1</td>
<td>0</td>
<td>(1/2 =) 0.5</td>
</tr>
<tr>
<td>Oliva superior</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

### Medulla oblongata (Block V)

<table>
<thead>
<tr>
<th></th>
<th>L</th>
<th>R</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>N. hypoglossus</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N. glossopharyngeus</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N. vestibularis</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N. trigeminus</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N. ambiguus</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Formatio reticularis</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Oliva inferior</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

### Cerebellum (Blocks IV and V)

<table>
<thead>
<tr>
<th></th>
<th>L</th>
<th>R</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>N. dentatus</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Other nuclei</td>
<td>0</td>
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</table>

### Spinal cord

<table>
<thead>
<tr>
<th></th>
<th>I</th>
<th>I</th>
<th>II</th>
<th>II</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L</td>
<td>R</td>
<td>L</td>
<td>R</td>
<td>L</td>
<td>R</td>
<td>L</td>
<td>L&amp;R</td>
</tr>
<tr>
<td>Cervical</td>
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<td></td>
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<td></td>
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</tr>
<tr>
<td>enlargementa,d</td>
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<td>3</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>2</td>
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<td>2</td>
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<td>2</td>
<td>5</td>
<td>11</td>
</tr>
<tr>
<td>Lumbar</td>
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<td>2</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>5</td>
</tr>
</tbody>
</table>

- ^a^ Discriminator area for rhesus.
- ^d^ Target area for cynomolgus.
Calculations:
Discriminator areas (globus pallidus, putamen, n. ant./med. thalami, n. lat. thalami):

\[
\text{Lesion score} = \frac{0.75 + 1.00 + 0.75 + 1.25}{4} = 0.94
\]

Target areas (s. nigra, cervical enlargement, lumbar enlargement):

\[
\text{Lesion score} = \frac{2.00 + 2.08 + 1.33}{3} = 1.80
\]

Discriminator plus target areas:

\[
\text{Lesion score} = \frac{0.94 + 1.80}{2} = 1.37
\]
### Summary of histopathology results

Date of inoculation: _________________  Species: _________________

<table>
<thead>
<tr>
<th>Monkey no.</th>
<th>Discriminator area score</th>
<th>Discriminator plus target area score</th>
<th>Monkey no.</th>
<th>Discriminator area score</th>
<th>Discriminator plus target area score</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
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<td></td>
</tr>
<tr>
<td><strong>Group mean</strong></td>
<td></td>
<td></td>
<td><strong>Group mean</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Result (pass or fail) ____________________________________________
Appendix 4

Example, for guidance, of cell culture techniques for the potency evaluation of yellow fever vaccine

Vero cells or pig kidney epithelial (PS) cells (1) may be used. (Note that PS cells are latently infected with swine fever virus and their importation is prohibited in certain countries.) Vero cell seed and a description of a method for Vero cell cultivation may be obtained from WHO.

A reference vaccine calibrated in IU should be included in all assays, with potency expressed as IU/dose.

Monolayers of the cell substrate are prepared in six-well (35 mm) tissue culture plates. Serial fourfold dilutions of the reconstituted test and reference vaccine are prepared and inoculated in duplicate in the plate wells and incubated at 36 °C for 1 h. After this incubation period, the inoculum is replaced by 3 ml of agarose or 3.3% carboxymethyl cellulose (CMC) overlay, and the plates are further incubated at 36 °C for 7 days. The agarose or CMC overlay is removed and the cell cultures are stained with either naphthalene black or crystal violet, washed and air-dried. The virus plaques are counted. In calculating the titre, all dilutions should be considered in which the average number of plaques per well is between 1 and 30. The potency in IU/dose is calculated relative to the standard vaccine.

For the test to be considered valid:

- the control cells should not show any plaque-forming or other cytopathic effect;
- the reference vaccine should be within 100.5 (0.5 log10) IU of its established mean titre.

Since yellow fever virus is light sensitive, the vaccine should be protected from direct light during storage and testing.

Further detailed guidance is available in the Manual of laboratory methods for testing vaccines used in the WHO Expanded Programme on Immunization (2).

References

Appendix 5

Model summary protocol for manufacturing and control of live attenuated yellow fever vaccines

The following protocol is intended for guidance. It indicates the minimum information that should be provided by the manufacturer to the NRA. Information and tests may be added or deleted as required by the NRA, if applicable.

It is possible that a protocol for a specific product may differ in detail from the model provided. The essential point is that all relevant details demonstrating compliance with the licence and with the relevant WHO recommendations on a particular product should be given in the protocol submitted.

The section concerning the final product must be accompanied by a sample of the label from the vaccine container and a copy of the leaflet that accompanies it. If the protocol is submitted in support of a request to permit importation, it should also be accompanied by a lot release certificate from the NRA of the country in which the vaccine was produced/released, stating that the product meets national requirements as well as Part A of the WHO Recommendations to assure the quality, safety and efficacy of live attenuated yellow fever vaccines.

Summary information on the finished product (final lot)

International name: _____________________________
Trade name: _____________________________
Product licence (marketing authorization) number: _____________________________
Country: _____________________________
Name and address of manufacturer: _____________________________
Site of manufacture of final lot: _____________________________
Name and address of licence-holder
(if different from manufacturer): _____________________________
Virus strain: _____________________________
Origin and short history: _____________________________
Authority that approved virus strain: _____________________________
Date approved: _____________________________
Final lot number: _____________________________
Final bulk number: _____________________________
Volume of final bulk: _____________________________
Final product: _____________________________
Type of container: _____________________________
Number of doses per container: _____________________________
Number of filled containers in this final lot: ______________________
Date of manufacture of final lot (filling or lyophilizing, if applicable): ______________________
Date on which last determination of virus concentration was started, or date of start of period of validity: ______________________
Shelf-life approved (months): ______________________
Expiry date: ______________________
Diluent: ______________________
Storage conditions: ______________________
Volume of single human dose: ______________________
Volume of vaccine per container: ______________________
Number of doses per container: ______________________
Prescribed virus concentration per single human dose: ______________________
Antibiotics added: ______________________
Release date: ______________________

Production information
A genealogy of the lot numbers of all vaccine components used in the formulation of the final product will be informative.

The following sections are intended for reporting the results of the tests performed during the production of the vaccine.

Starting materials
The information requested below is to be presented on each submission. Full details on master and working seed lots should be provided upon first submission only and whenever a change has been introduced.

Virus master seed lot
Source of 17D substrain: ______________________
Master virus seed lot number: ______________________
Name and address of manufacturer: ______________________
Passage level: ______________________
Date of inoculation of embryos: ______________________
Date of harvest: ______________________
Age of embryos (at harvest): ______________________
Number of containers: ______________________
Conditions of storage: ______________________
Date virus master seed lot was established: ______________________
Date approved by the NRA: ______________________
Information on source materials

Source of eggs ____________________________

Is the flock under direct control of the manufacturer? ____________________________

Is the flock monitored for compliance with these recommendations? ____________________________

Tests on virus master seed lot production (A.4.2.2)

Identity test (A.4.2.2.1)

Method used: ____________________________

Date test started: ____________________________

Date test ended: ____________________________

Results: ____________________________

Lot number of Reference Reagents: ____________________________

Genotype characterization (A.4.2.2.2)

Method used: ____________________________

Dates test started and ended: ____________________________

Results: ____________________________

Lot number of Reference Reagents: ____________________________

Freedom from bacteria, fungi and mycoplasmas (A.4.2.2.3)

Tests for bacteria and fungi

Method used: ____________________________

Number of vials tested: ____________________________

Volume of inoculum per vial: ____________________________

Volume of medium per vial: ____________________________

Observation period (specification): ____________________________

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Media used</th>
<th>Inoculum</th>
<th>Date test began</th>
<th>Date test ended</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>20–25 °C</td>
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<td></td>
<td></td>
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Tests for mycoplasmas

Method used: ____________________________

Volume tested: ____________________________

Media used: ____________________________
Temperature of incubation: ________________________________
Observation period (specification): ________________________________
Positive controls (list of species used and results): ________________________________

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<td>Subcultures at day 21</td>
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</table>

*Indicator cell culture method (if applicable)*

- Cell substrate used: ________________________________
- Inoculum: ________________________________
- Date of test: ________________________________
- Passage number: ________________________________
- Negative control: ________________________________
- Positive controls: ________________________________
- Date of staining: ________________________________
- Results: ________________________________

*Tests for ALVs and other adventitious agents (A.4.2.2.4)*

- Method: ________________________________
- Volume tested: ________________________________
- Date test started: ________________________________
- Date test ended: ________________________________
- Result: ________________________________

*Tests for avian mycobacteria*

- Method: ________________________________
- Media used: ________________________________
- Temperature of incubation: ________________________________
- Volume tested: ________________________________
- Date test started: ________________________________
- Date test ended: ________________________________
- Result: ________________________________

*Safety test on animals (guinea-pigs, mice and embryonated chicken eggs)*

- Species used: ________________________________
- Number of animals inoculated: ________________________________
- Volume injected per animal: ________________________________
Inoculation route: ___________________________________________________________________
Date test started: ___________________________________________________________________
Date test ended: ___________________________________________________________________
Result: _____________________________________________________________________________

Testing in nonhuman primates (A.4.2.2.5)
See Appendix 2

Virus titration for infectivity (A.4.2.2.6)
Method: __________________________________________________________________________
Date: _____________________________________________________________________________
Result: ___________________________________________________________________________

Virus working seed lot
Working virus seed lot number: ___________________________________________________________________
Name and address of manufacturer: ___________________________________________________________________
Passage level: __________________________________________________________________________
Date of inoculation of embryos: ___________________________________________________________________
Temperature of incubation: ___________________________________________________________________
Date of harvest: __________________________________________________________________________
Age of embryos (at harvest): ___________________________________________________________________
Date of filling: __________________________________________________________________________
Date of lyophilization (if appropriate): ___________________________________________________________________
Number of containers: __________________________________________________________________________
Conditions of storage: __________________________________________________________________________
Date virus working seed lot was established: ___________________________________________________________________
Date approved by the NRA: __________________________________________________________________________

Information on source materials
Source of eggs: __________________________________________________________________________

Is the flock under direct control of manufacturer? ___________________________________________________________________

Is the flock monitored for compliance with these recommendations? ___________________________________________________________________

Tests on virus working seed lot production (A.4.2.2)
Identity test (A.4.2.2.1)
Method used: __________________________________________________________________________
Date test started: __________________________________________________________________________
Date test ended: __________________________________________________________________________
Results: ____________________________
Lot number of Reference Reagents: ____________________________

Genotype characterization (A.4.2.2.2)
Method used: ____________________________
Date test began and ended: ____________________________
Results: ____________________________
Lot number of Reference Reagents: ____________________________

Freedom from bacteria, fungi and mycoplasmas (A.4.2.2.3)

Tests for bacteria and fungi
Method used: ____________________________
Number of vials tested: ____________________________
Volume of inoculum per vial: ____________________________
Volume of medium per vial: ____________________________
Observation period (specification): ____________________________

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<tr>
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<th>Media used</th>
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<td>Negative control</td>
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Tests for mycoplasmas
Method used: ____________________________
Volume tested: ____________________________
Media used: ____________________________
Temperature of incubation: ____________________________
Observation period (specification): ____________________________
Positive controls (list of species used and results): ____________________________

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</tbody>
</table>
Indicator cell culture method (if applicable)

- Cell substrate used: ____________________________
- Inoculum: ____________________________
- Date of test: ____________________________
- Passage number: ____________________________
- Negative control: ____________________________
- Positive controls: ____________________________
- Date of staining: ____________________________
- Results: ____________________________

Tests for ALVs and other adventitious agents (A.4.2.2.4)

- Method: ____________________________
- Volume tested: ____________________________
- Date test started: ____________________________
- Date test ended: ____________________________
- Result: ____________________________

Tests for avian mycobacteria

- Method: ____________________________
- Media used: ____________________________
- Temperature of incubation: ____________________________
- Volume tested: ____________________________
- Date test started: ____________________________
- Date test ended: ____________________________
- Result: ____________________________

Tests for other adventitious agents on cell culture

Human diploid cells/monkey kidney cells/primary chick embryo fibroblast cells

- Method used: ____________________________
- Test on cell culture: ____________________________
- Type of cells: ____________________________
- Cell strain: ____________________________
- Lot number of antiserum: ____________________________
- Volume tested: ____________________________
- Temperature of incubation: ____________________________
- Date test started: ____________________________
- Date test ended: ____________________________
- Date of haemadsorption (if applicable): ____________________________
- Result: ____________________________
Test for adventitious agents on eggs (avian viruses)

**Allantoic cavity**

Lot number of antiserum: ____________________________
Number of eggs inoculated: __________________________
Volume inoculated per egg: __________________________
Temperature of incubation: _________________________
Inoculation date: _________________________________
Date of harvest: _________________________________
Date of haemagglutination test: ____________________
Result: _________________________________________

**Yolk sac**

Number of eggs inoculated: __________________________
Volume inoculated per egg: __________________________
Temperature of incubation: _________________________
Inoculation date: _________________________________
Date of collection of embryo for observation: __________
Result: _________________________________________

Safety test on animals (guinea-pigs, mice and embryonated chicken eggs)

Species used: _________________________________
Number of animals inoculated: ______________________
Volume injected per animal: _______________________
Inoculation route: _______________________________
Date test started: _______________________________
Date test ended: _______________________________
Result: _________________________________________

Testing in nonhuman primates (A.4.2.2.5)

See Appendix 2

Virus titration for infectivity (A.4.2.2.6)

Method: _______________________________________
Date: __________________________________________
Result: _________________________________________

Control of vaccine production (A.5)

*Information on source materials* ________________________________
Source of eggs: __________________________________________
Is the flock under direct control of manufacturer? _________
Is the flock monitored for compliance with these recommendations?  

**Virus used to inoculate embryos**
- Derived from master seed virus lot number: 
- Working virus seed lot, reference number and source: 
- Passage level of working virus seed lot: 

**Information on manufacture**
- Date of inoculation of embryos: 
- Quantity of inoculated embryos: 
- Temperature of incubation: 
- Date of harvest: 
- Age of embryos (at time of harvest): 
- Quantity of harvested embryos: 
- Number of rejected eggs (ratio): 
- Number of containers: 
- Conditions of storage: 
- Expiry date: 

**Tests on uninoculated control eggs (A.5.1)**
- Number of eggs used: 

**Test for haemagglutinating agents**

**Directly on allantoic fluid**
- Method: 
- Volume tested: 
- Date of test: 
- Result: 

**After a passage in SPF eggs**
- Method: 
- Volume tested: 
- Route of inoculation: 
- Date test started: 
- Date test ended: 
- Result: 

**Test for other adventitious agents on cell culture**
- Human diploid cells/monkey kidney cells/primary chick embryo fibroblast cells
Cell type: __________________________
Volume tested: __________________________
Temperature of incubation: __________________________
Date test started: __________________________
Date test ended: __________________________
Result: __________________________

Test for ALVs
Method: __________________________
Volume tested: __________________________
Date test started: __________________________
Date test ended: __________________________
Result: __________________________

Tests on control tissues
Test for Salmonella
Method: __________________________
Volume tested: __________________________
Date test started: __________________________
Date test ended: __________________________

Tests for avian mycobacteria
Method: __________________________
Media used: __________________________
Temperature of incubation: __________________________
Volume tested: __________________________
Date test started: __________________________
Date test ended: __________________________
Result: __________________________

Test for fowl pox virus
Method: __________________________
Volume tested: __________________________
Volume of negative controls: __________________________
Date test started: __________________________
Date test ended: __________________________
Result: __________________________

Tests for ALVs (if applicable)
Method used: __________________________
Volume tested: ____________________________
Temperature: ____________________________
Date test started: ________________________
Date test ended: _________________________
Result: __________________________________________________________________________

Test for haemagglutinating agents on embryonated hen eggs (avian viruses)

Allantoic cavity
- Number of eggs inoculated: ____________________________
- Volume inoculated per egg: ____________________________
- Temperature of incubation: ____________________________
- Inoculation date: ________________________________
- Date of harvest: ________________________________
- Date of haemagglutination test: _____________________
- Result: __________________________________________________________________________

Yolk sac
- Number of eggs inoculated: ____________________________
- Volume inoculated per egg: ____________________________
- Temperature of incubation: ____________________________
- Inoculation date: ________________________________
- Date of collection of embryo for observation: __________________
- Result: __________________________________________________________________________

Test for other extraneous agents on cell culture
Human diploid cells/monkey kidney cells/primary chick embryo fibroblast cells

- Cell type: __________________________
- Volume tested: ____________________________
- Temperature of incubation: __________________
- Date test started: ________________________
- Date test ended: _________________________
- Result: __________________________________________________________________________

Tests on single harvests (A.5.3)

Identity test (A.5.3.2) __________________________________________________________________
- Date test started: ________________________
- Date test ended: _________________________
- Result: __________________________________________________________________________
**Freedom from bacteria, fungi and mycoplasmas (A.5.3.3)**

**Tests for bacteria and fungi**

Method used: 

Number of vials tested: 

Volume of inoculum per vial: 

Volume of medium per vial: 

Observation period (specification): 

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<tr>
<th>Incubation</th>
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<td>Negative control</td>
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</table>

**Tests for mycoplasmas**

Method used: 

Volume tested: 

Media used: 

Temperature of incubation: 

Observation period (specification): 

Positive controls (list of species used and results): 

<table>
<thead>
<tr>
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<tr>
<td>Subcultures at day 21</td>
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</tbody>
</table>

**Indicator cell culture method (if applicable)**

Cell substrate used: 

Inoculum: 

Date of test: 

Passage number: 

Negative control: 

Positive controls: 

Date of staining: 

Results: 
Tests for adventitious agents (A5.3.4)

Tests for Mycobacterium avium
Method: 
Media used: 
Temperature of incubation: 
Volume tested: 
Date test started: 
Date test ended: 
Result: 

Virus titration (A.5.3.5)
Method: 
Date: 
Result: 

Control of final bulk (A.5.4)
Sterility tests (A.5.4.1)
Tests for bacteria and fungi
Method used: 
Number of vials tested: 
Volume of inoculum per vial: 
Volume of medium per vial: 
Observation period (specification): 

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<tr>
<th>Incubation</th>
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Stabilizers (if added) (A.5.4.2)
Name of stabilizer: 
Quantity or percentage: 
Date: 

Virus titration (if performed) (A.5.4.3)
Method: 
Date: 
Result: 
**Filling and containers (A.6)**

Lot number: _____________________________

Date of filling: ____________________________

Volume of final bulk filled: ____________________________

Filling volume per container: ____________________________

Number of containers filled (gross): ____________________________

Date of lyophilization: ____________________________

Number of containers rejected during inspection: ____________________________

Number of containers sampled: ____________________________

Total number of containers (net): ____________________________

Maximum period of storage approved: ____________________________

Storage temperature and period: ____________________________

**Control tests on final lot (A.7)**

**Inspection of final containers (A.7.1)**

Appearance: ____________________________

Date of test: ____________________________

Results: ____________________________

Before reconstitution: ____________________________

After reconstitution: ____________________________

Diluent used: ____________________________

Lot number of diluent used: ____________________________

**Identity test (A.7.2)**

Method used: ____________________________

Date test started: ____________________________

Date test ended: ____________________________

Results: ____________________________

Lot number of Reference Reagents: ____________________________

**Potency test (A.7.3)**

Date of test: ____________________________

Reference batch number: ____________________________

Specification: ____________________________

Titre of reference batch (IU/0.5 ml): ____________________________

Vaccine: Virus concentration (IU/human dose)

<table>
<thead>
<tr>
<th>Vial</th>
<th>Virus concentration (IU/human dose)</th>
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<td>2</td>
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<tr>
<td>3</td>
<td>____________________________</td>
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</table>

Mean virus titre per human dose, with 95% fiducial limits: ____________________________
Thermal stability test (A.7.4)

Date of test: 
Reference batch number: 
Titre of reference batch (IU/0.5 ml): 
Vaccine held at 37 °C for 14 days: 
Vaccine: Virus concentration (IU/human dose)
  Vial 1: 
  Vial 2: 
  Vial 3: 
Mean virus titre per human dose, with 95% fiducial limits: 
Loss in titre (in log10 IU): 

Sterility tests (A.7.5)

Tests for bacteria and fungi
Method used: 
Number of vials tested: 
Volume of inoculum per vial: 
Volume of medium per vial: 
Observation period (specification): 

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General safety test (if performed) (A.7.6)

Tests in mice
Date of inoculation: 
Number of mice tested: 
Volume and route of injection: 
Observation period: 
Results (give details of deaths): 

Tests in guinea-pigs
Date of inoculation: 
Number of guinea-pigs tested: 
Volume and route of injection: 
Observation period: 
Results (give details of deaths): 
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Method</th>
<th>Specification</th>
<th>Date</th>
<th>Result</th>
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<td>Residual moisture (A.7.7)</td>
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<td>Residual ovalbumin (A.7.8)</td>
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<td>Endotoxin (A.7.9)</td>
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<td>Residual antibiotics (if applicable) (A.7.10)</td>
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</table>

**Submission addressed to national regulatory authority**

Name of Head of Production (typed) ________________________________

Certification by the person from the control laboratory of the manufacturing company taking over responsibility for the production and control of the vaccine:

I certify that lot no. _______________ of yellow fever vaccine, whose number appears on the label of the final container, meets all national requirements and/or satisfies Part A of the WHO Recommendations to assure the quality, safety and efficacy of live attenuated yellow fever vaccines.

Signature: ________________________________
Name (typed): ________________________________
Date: ________________________________
Appendix 6

Model certificate for the release of live attenuated yellow fever vaccine by a national regulatory authority

LOT-RELEASE CERTIFICATE

The following lot(s) of yellow fever vaccine produced by __________________________ in __________________________, whose numbers appear on the labels of the final containers, meet all national requirements and Part A of the WHO Recommendations to assure the quality, safety and efficacy of live attenuated yellow fever vaccines (________________________), and comply with WHO Good manufacturing practices: main principles for pharmaceutical products and Good manufacturing practices for biological products.

As a minimum, this certificate is based on examination of the summary protocol of manufacturing and control.

The certificate may include the following information:

- name and address of manufacturer;
- site(s) of manufacturing;
- trade name and/or common name of product;
- marketing authorization number;
- lot number(s) (including sub-lot numbers and packaging lot numbers if necessary);
- type of container;
- number of doses per container;
- number of containers/lot size;
- date of start of period of validity (e.g. manufacturing date) and/or expiry date;

1 Name of manufacturer.
2 Country of origin.
3 If any national requirements are not met, specify which one(s) and indicate why release of the lot(s) has nevertheless been authorized by the national regulatory authority.
4 With the exception of provisions on distribution and shipping, which the national regulatory authority may not be in a position to assess.
- storage condition;
- signature and function of the authorized person and authorized agent to issue the certificate;
- date of issue of certificate;
- certificate number.

The Director of the national regulatory authority (or authority as appropriate):

Name (typed) ______________________________
Signature _________________________________
Date _________________________________
Annex 6

Procedure for assessing the acceptability, in principle, of vaccines for purchase by United Nations agencies

Abbreviations
1. Introduction
2. Conditions for acceptance of applications
3. Steps of the procedure
   3.1 Official request and response
   3.2 Meetings with manufacturers
   3.3 Product summary file
   3.4 Initial testing of vaccine samples
   3.5 WHO site audits
   3.6 Report and outcome of the assessment
4. Considerations for streamlining the prequalification procedure on the basis of enhanced assistance by NRAs
   4.1 Procedure for selecting eligible NRAs
   4.2 Streamlined procedure for vaccines with marketing authorization/licensing granted by eligible NRAs
   4.3 Vaccines with positive scientific opinion issued by the EMA
5. Special considerations for fast-track procedure
6. Special considerations for accepting submissions of vaccines manufactured at multiple sites or in different countries
7. Obligations after prequalification is granted
8. Annual reporting
9. Reassessments
10. Monitoring continued compliance with specifications through targeted testing
11. Monitoring vaccine quality complaints or AEFIs from the field
    11.1 Vaccine quality complaints
    11.2 AEFIs
12. Recommendations for action in cases of non-compliance
13. Handling out-of-specification/inconsistent results between laboratories
This document is intended to be scientific and advisory. Each of the following sections constitutes guidance for manufacturers of biological products. The parts of each section printed in small type are comments for additional guidance intended for manufacturers, which may benefit from these details.
Abbreviations

AEFI adverse event following immunization
AMC Advance Market Commitment
CHMP Committee for Medicinal Products for Human Use
CTD common technical document
EMA European Medicines Agency
GCP good clinical practice
GLP good laboratory practice
GMP good manufacturing practices
ICH International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
NRA national regulatory authority
NCL national control laboratory
OMCL official medicine control laboratory
PSF product summary file
PSPQ SC Programmatic Suitability of Vaccines for Prequalification (Standing Committee)
PSUR periodic safety updated report
UNICEF United Nations Children’s Fund
USA United States of America
VVM vaccine vial monitor

1. Introduction

The World Health Organization (WHO), through its Department of Immunization, Vaccines and Biologicals, provides advice to the United Nations Children’s Fund (UNICEF) and other United Nations agencies on the acceptability, in principle, of vaccines considered for purchase by such agencies. This service is called prequalification. The purpose of the United Nations prequalification assessment is to provide assurance that candidate vaccines: (a) meet WHO recommendations on quality, safety and efficacy, including compliance with WHO’s recommended standards for good manufacturing practices (GMP) and good clinical practice
(GCP); and (b) meet the operational packaging and presentation specifications of the relevant United Nations agency. The aim is to ensure that vaccines provided through the United Nations for use in national immunization services in different countries are safe, effective and suitable for the target populations at the recommended immunization schedules and with appropriate concomitant products.

The procedure in place at WHO to assess the acceptability of candidate vaccines for the United Nations was published initially in the thirty-ninth report of the WHO Expert Committee on Biological Standardization (1). Since then, a number of published revisions to the procedure have been implemented (in 1996, 2002 and 2005; 2).1

The current document is a revision that takes into consideration challenges faced by the vaccines prequalification programme – such as the increasing number of submissions and the increasing diversity and complexity of the products submitted to WHO for evaluation, as well as the ongoing maintenance of the prequalified status for those vaccines on the list. The latter includes reassessments and reviews of variations, and investigation of quality and safety concerns reported by fieldworkers, which equate to a growing workload for WHO.

This document addresses technical, communication and policy aspects of the procedure and is based on the recommendations made by an Ad Hoc Advisory Committee of Experts on Vaccines Prequalification convened by WHO in May 2010, and on a series of supporting documents. The document proposes an update of the current procedure.

The prequalification procedure established by WHO for vaccines has been effective in promoting confidence in the quality of the vaccines shipped to countries through United Nations purchasing agencies. The procedure is based on the following principles:

- reliance on the national regulatory authority (NRA) of the country of manufacture, which is required to be “functional”, i.e. meeting the published WHO NRA indicators for prequalification purposes (3);
- general understanding of the product and presentations offered, the production process, quality control methods, quality system in place, and available clinical data that are relevant to the target population;
- assurance of production consistency through compliance with GMP requirements and monitoring of continued compliance with specifications through testing of final product characteristics.

WHO is able to advise United Nations agencies as to whether vaccines effectively meet the Organization’s recommended requirements only if the

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1 The revisions published in 1996 and 2002 were superseded by those published in 2005 and are therefore not available.
responsible NRA exercises independent and appropriate regulatory oversight of the vaccines in question and if the vaccines have been assessed through the procedure described in this document. Since reliance on effective regulatory oversight by the NRA of the country of manufacture plays a critical role in the system, manufacturers shall: (a) inform the NRA of their application to WHO for the vaccine prequalification by sending to the NRA a copy of the application letter sent to WHO; (b) request the NRA to participate/collaborate in the process; and (c) provide the NRA with the necessary authorization to discuss the relevant files with WHO representatives.

This update introduces a procedure for using, in certain circumstances, enhanced assistance from eligible NRAs (see section 4).

Under exceptional circumstances, extraordinary temporary measures may be applied in the situation where the NRA responsible for the regulatory oversight of a product fails to sustain its functionality with regard to WHO standards. Such measures are taken only where it is necessary to ensure a global supply of vaccines of assured quality. This procedure is applied to vaccines for which there is no immediate alternative source and where removal from the prequalified list would jeopardize the global supply.

As vaccines purchased by United Nations agencies are required to meet WHO recommendations or guidelines (whichever are available), novel vaccines for which such recommendations are not available cannot be evaluated. In cases where a vaccine is made available for a disease of public health importance, the development of such guidelines will be prioritized by WHO and, as soon as a draft document becomes available, this can be used for evaluation for prequalification purposes. The fact that certain vaccines are not included on the list of prequalified vaccines does not mean that, if evaluated, they would be found not to comply with the required standards. The database of prequalified vaccines can be consulted on the WHO web site (4).

WHO will define, in consultation with United Nations purchasing agencies, which vaccines are priorities for prequalification, and will make this information publicly available. Information on priority-setting for WHO vaccine prequalification is available on the WHO web site (5).

This exercise is required in order to focus the use of resources. Priorities are redefined at regular intervals, to ensure that efforts are put into evaluating those available vaccines that are of highest public health importance and most needed in developing countries.

2. Conditions for acceptance of applications

The conditions for acceptance of applications are as follows.

- The candidate vaccine is on the current list of priority products for United Nations prequalification.
The candidate vaccine meets the mandatory characteristics for programmatic suitability, as defined in the document *Assessing the programmatic suitability of vaccine candidates for WHO prequalification* (6).

WHO encourages manufacturers to discuss any concerns about programmatic suitability characteristics for prequalification with the prequalification secretariat, early in the development process.

The NRA responsible for the regulatory oversight of the product has been assessed by WHO as “functional” and has been found to meet all the critical indicators defined for prequalification purposes.

An applicant should check with the respective NRA whether it has been assessed by WHO. WHO will not be able to process an application until the WHO NRA assessment is conducted and the outcome is satisfactory.

A marketing authorization has been granted by the relevant NRA and the post-marketing regulatory oversight is under the responsibility of the NRA of the country of manufacture (or the European Medicines Agency (EMA) in the case of the centralized procedure for marketing authorizations in Europe) or that of the country of finishing and distribution. Alternatively, if it is intended that the EMA “scientific opinion”\(^1\) should serve as a basis to facilitate the marketing authorization of the vaccine, the *Guideline on procedural aspects regarding a CHMP [Committee for Medicinal Products for Human Use] scientific opinion in the context of cooperation with the World Health Organization* (7) should be used.

WHO encourages manufacturers to discuss the product and the regulatory requirements with the prequalification secretariat, early in the development process.

### 3. Steps of the procedure

For the evaluation of vaccines, WHO requires information related to the manufacturing company and to the product itself. The manufacturer will provide this information in the product summary file (PSF, see Appendix 1) and during

\(^1\) EMA scientific opinion, in accordance with Article 58 of Regulation (EC) No. 726/2004, is restricted exclusively to medicinal products that are not authorized within the European Union. However, the issuing of a scientific opinion does not prevent submission of a future European Union marketing authorization.
the site audit, if applicable. However, WHO reserves the right to terminate the assessment if at any time it is considered that insufficient information has been provided to enable effective completion of the assessment. The steps of the prequalification procedure are shown in Appendix 2.

3.1 **Official request and response**

An application letter\(^1\) is to be sent to the Coordinator, Quality, Safety and Standards, Department of Essential Medicines and Health Products in WHO, with copies to the vaccines prequalification manager and the relevant NRA, giving details of country and sites of manufacture, licensing status and the presentations put forward to United Nations agencies for procurement.

Application letters can be sent at any time and should provide the expected date of file submission.

To facilitate planning, manufacturers are encouraged to advise WHO as early as possible of their intention to submit a specific vaccine for evaluation.

WHO will acknowledge receipt and acceptance of the application letter by e-mail, with a copy to the NRA, and will respond with an official letter only in those cases where the vaccine will not be accepted because it is not a priority. In such cases, the applicant and the NRA will be advised of the rejection of the application within 2 weeks of receipt of the official request.

3.2 **Meetings with manufacturers**

If considered necessary or desirable by either party, a discussion may be held between the manufacturer, the responsible NRA (if willing to participate) and WHO before the actual evaluation process starts. This pre-evaluation meeting should be scheduled as early as possible, with a predefined agenda addressing questions sent to WHO in advance by the manufacturer.

Such meetings are important for discussing programmatic suitability issues and can be scheduled when requested by the manufacturer.

Additional meetings may be held during the evaluation process, as required.

3.3 **Product summary file**

A manufacturer whose application letter is accepted will prepare and submit one hard copy and five electronic copies (on CD-ROM), in either Microsoft Word or

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\(^1\) The purpose of the application letter is to communicate to WHO the manufacturer’s intention of submitting a vaccine for evaluation.
PDF format, of a product summary file (PSF), which should be fully up to date and written entirely in English following the WHO format provided below:

- Chapter 1: General information;
- Chapter 2: Personnel;
- Chapter 3: Premises and equipment;
- Chapter 4: Vaccine composition, presentations and schedules;
- Chapter 5: Production;
- Chapter 6: Quality control;
- Chapter 7: Stability;
- Chapter 8: Clinical experience;
- Chapter 9: Production and distribution data;
- Chapter 10: Update on regulatory actions.

The WHO format is required; however, the common technical document (CTD) format can be accepted so long as (a) a detailed cross-referencing of contents is presented; and (b) those aspects required by WHO but not included in the CTD requirements are presented. Where the PSF cross-references to the CTD format, the documentation may be in electronic form only. Electronic documents should be in searchable text where possible.

The information to be provided in the file is specified in Appendix 1 of this document.

WHO has established three deadlines per year for the submission of PSFs: 31 January, 31 May and 30 September.

In each case, applications must arrive at WHO by the submission date, in order to be considered for the subsequent round of review. Applications received after the submission deadline will not be considered for evaluation until the following review round.

3.3.1 Screening of the PSF and payment

Upon receipt, the PSF will be screened for completeness and compliance with the required format and contents. If the PSF is not in compliance with the format and contents, the manufacturer will be informed through an official letter and required to pay the screening fees. An improved PSF may be submitted for a subsequent scheduled submission deadline without additional payment. In the case of a second (final) rejection, the manufacturer will be informed by official letter and an invoice will be sent requesting payment of the screening fees.

In addition, an assessment of the suitability of the vaccine for the immunization services in the location where it is intended to be used will also be conducted at this stage. The process for review of programmatic suitability of
vaccine characteristics is described in the document *Assessing the programmatic suitability of vaccine candidates for WHO prequalification* (6).

At the time of screening, vaccine candidates must be in compliance with the mandatory programmatic characteristics\(^1\) as defined by WHO’s Immunization Practices Advisory Committee. If screening reveals that the mandatory characteristics are not met, then the PSF will be rejected. If the prequalification secretariat identifies a deviation from the critical characteristics or a unique, novel and innovative characteristic, as defined by WHO (6), a recommendation from the Programmatic Suitability for Prequalification (PSPQ) Standing Committee is required.

The PSPQ Standing Committee is an advisory body to the prequalification secretariat and the director of the Department of Immunization, Vaccines and Biologicals. The Standing Committee consists of experts on immunization programmes and vaccines regulation. The terms of reference of the PSPQ Standing Committee are available in reference 8.

The Standing Committee will review the documentation exclusively related to the specific problem. During its review and discussion, which will lead to the formulation of recommendations, the PSPQ Standing Committee may engage in confidential discussion with manufacturers and other technical experts approved by WHO and the manufacturer. All members of the PSPQ Standing Committee will be required to sign a confidentiality agreement (see section 15 and Appendix 3) and a declaration of interests form (see section 16 and Appendix 4) prior to taking up their responsibilities.

Under special circumstances, when there is limited access to a vaccine of public health importance, exceptional consideration will be given regarding the suitability of vaccine candidates that are non-compliant with the critical characteristics or that present with unique and innovative characteristics. This decision can be made by the prequalification secretariat and will take into account the recommendations of the PSPQ Standing Committee, public health needs and availability of alternative products.

The screening process will be put on hold while the PSPQ Standing Committee conducts the review. The duration of the review by the PSPQ Standing Committee will be no longer than 3 months. In case of rejection following a recommendation from the PSPQ Standing Committee, the reviewers may include a recommendation for resubmission after validation by research of the acceptability of specific vaccine characteristics.

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\(^1\) “Mandatory” characteristics are those where compliance is compulsory at the time of application for WHO prequalification and must be unconditionally met prior to evaluation of the PSF (see reference 6).
When no review by the PSPQ Standing Committee is required, the manufacturer will be informed within 1 month from the submission deadline if the PSF is accepted for further review or rejected. In case of acceptance, the manufacturer will be informed by letter of the acceptance of the file for evaluation and of the names of the experts\(^1\) proposed for the evaluation, together with a copy of their curricula vitae. At the same time, an invoice will be sent by WHO requesting payment of the screening and evaluation fees. Manufacturers will be expected to pay the fee and confirm the acceptability of the proposed experts within 2 weeks. Payment of the fees without any further communication will be considered as de facto agreement to the proposed experts. The evaluation will then be initiated.

In case of rejection for any reason, the manufacturer will be informed through an official letter, and an invoice will be sent by WHO requesting payment of the screening fees. With the agreement of the manufacturer, the PSF will be destroyed by WHO.

### 3.3.2 PSF evaluation

The time frame for an initial review of a vaccine PSF will be 3 months. A consolidated report will be provided to manufacturers, who are expected to submit responses to comments and any complementary information that may be requested. WHO takes no further action until the full complementary information is received.

The complementary information must be submitted in a single package containing one hard copy and five electronic copies with, adequate cross-referencing to the original file. If partial responses are received at different times, the review will not start until all of the outstanding items have been addressed by the manufacturer.

WHO reserves the right to terminate this procedure for a specific vaccine if the manufacturer is not able to provide the required response with an acceptable action plan within 3 months and the actual information within the agreed time period, or if the information supplied is inadequate.

The time frame for review of complete complementary information will be 3 months.

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\(^1\) NRA staff, independent consultants or staff from consulting companies may be appointed as external experts, depending on the specific needs. The manufacturer has the right to reject one or more team members if justification is provided, in which case WHO will find a replacement. All experts appointed by WHO to participate in the evaluation of a vaccine are required to sign a confidentiality agreement (see section 15 and Appendix 3) and a declaration of interests form (see section 16 and Appendix 4) for that specific evaluation.
3.4 Initial testing of vaccine samples

As soon as the PSF is accepted and when the prequalification procedure described in section 3.3 is applied, WHO will request the manufacturer to submit an appropriate number of samples (between 25 and 200, depending on the vaccine type and presentation offered) of three to five final lots for independent testing. These lots will have been formulated from consecutive bulk lots (in the case of combination vaccines, consecutive bulks will be specified by WHO for one of the components).

The samples should be accompanied by the respective lot summary protocols, fully detailed as described in the WHO Guidelines for independent lot release of vaccines by regulatory authorities (9) and the detailed standard operating procedure for testing the product characteristics (relevant tests). Biological reagents and reference materials for the validation of the tests by WHO-contracted laboratories should be provided by the manufacturer. In some cases, samples of bulk material may be requested.

WHO will send the vaccine samples to the contracted laboratories for the initial testing. Tests undertaken will be the most relevant to reflect the quality, safety and efficacy of the vaccine. Usually, potency and toxicity are tested. However, depending on the nature of the vaccines, other relevant tests may be performed. If applicable, the relevant method should be transferred from the manufacturer to the contracted laboratory through WHO. The performance of the contracted laboratories in conducting the relevant tests is evaluated by WHO.

The samples subject to testing must comply in all respects with the information and specifications stated in the PSF. They must have been produced under full-scale production conditions, and must be representative samples of the product that is intended for marketing through United Nations agencies. The expected time frame for testing, from the date of receipt of the samples by WHO to the finalization of testing by WHO, is 3 months.

To promote the independence and impartiality of the testing, neither the manufacturer nor any other party who may have requested that vaccines be tested through this system will be informed of where the testing is performed. Situations where the manufacturer is asked by WHO to transfer the testing methodology to a national control laboratory (NCL) will be the exception to this rule. Upon request, the manufacturer and the relevant NRA will, however, receive a report of the test results.

In general the selected contracted laboratories do not include the NCL of the NRA in charge of the testing for lot release. Exceptions can be made in the case of a streamlined procedure.

3.5 WHO site audits

The main objectives of site audits are to assess whether the vaccine complies with WHO recommendations for production and quality control, whether
it meets the United Nations’ specifications for tender (which reflect the needs of the immunization programmes at country level), whether the company has an adequate quality system in place, and whether the vaccine is produced in compliance with WHO-recommended GMP.\(^1\) Other important aspects of the assessment include, but are not limited to, labelling, packaging, whether a post-marketing surveillance system is in place, vaccine vial monitor (VVM) implementation when required, and a stability programme.

Site audits are required for those manufacturers applying for the prequalification of new products to be evaluated for purchase by United Nations agencies. They are necessary as part of the initial evaluation, as follow-up to corrective actions taken by the manufacturer following WHO recommendations, and for reassessment purposes. They may also be deemed necessary as a result of complaints or reports of serious adverse events following immunization (AEFIs) if a quality problem is suspected.

Site audits are part of the standard assessment performed to ensure that vaccine candidates for purchase by United Nations agencies (or those that are already being purchased) meet (or continue to meet) WHO recommendations and tender specifications. As far as possible, site audits build on information gathered through inspections performed by NRAs that meet the critical indicators established by WHO for vaccine prequalification purposes. In such cases, if detailed reports of inspections are made available for WHO review, WHO may decide, in agreement with the manufacturer, to organize an abbreviated site audit. This would focus only on aspects relevant to the product under evaluation that have not been addressed by the NRA that did the inspection, including all those aspects that are specific to the United Nations tender specifications.

For a new application, when the review of the PSF and testing have been satisfactorily completed, WHO may assemble a team to audit the manufacturing facility. The site audit will take place as soon as possible after satisfactory test results are available, and usually within 2 months. Technical staff from the relevant United Nations agency may elect to join the team. Otherwise, the team will be composed, as far as possible, of the experts who reviewed the file. Team members must have expertise in the areas of production, quality control, quality assurance, quality system and GMP. If additional members or replacement members are needed, the curricula vitae of the proposed new members will be submitted to the company for clearance. The team will cover the range of expertise required to assess the vaccine in question from the different perspectives. A WHO staff member will lead the audit team and the members will act, on a temporary basis.

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\(^1\) With regard to aspects for which GMP requirements are not sufficiently detailed, other international guidelines should be followed by the manufacturer and appropriate justification for the choice provided. In such cases, WHO will assess against the standard used.
as expert advisers to WHO. In some circumstances, leadership can be delegated to one of the external experts, who will act on behalf of WHO.

The NRA of the manufacturing country, or the NRA with regulatory oversight of the product, will be invited to assign one or two staff members to join the WHO team as observers.

A bilateral consultation meeting will be held between WHO and the NRA, either at the beginning or at the end of the mission. The purpose of this meeting is to discuss regulatory matters related to the vaccine in question and to lay the basis for the letters of agreement. Topics addressed during such consultation meetings relate to commitment for testing and release of vaccine lots for United Nations agencies, the need for feedback on findings during inspections, updates on safety and efficacy data, variations to the marketing authorization/licence that may have been requested, marketing authorization/licence renewals, recalls or withdrawal of lots, etc. WHO will establish letters of agreement with all the NRAs responsible for the oversight of prequalified products.

WHO site audits of manufacturing facilities or results of consultations held with the NRA may trigger a follow-up assessment of the NRA for one or more functions. In such cases, the follow-up assessments should be performed within no more than 6 months. The outcome of the follow-up assessment may have an impact on the final decision about the prequalification of the vaccines in question. The findings and recommendations of the team will be discussed with the company on a daily basis, as required during the site audit. Where relevant, the team may request the manufacturer to prepare a corrective action plan to address critical recommendations and establish deadlines for receiving responses. The draft report, which includes the main findings, recommendations and closing remarks, is prepared by the WHO team and left with the manufacturer. The findings and recommendations will also be reported to company and NRA representatives during the closing meeting, thus providing an opportunity for discussion, questions and clarifications.

The final report with findings, recommendations and conclusions is prepared by the team and sent to the company, with a copy to the NRA, within 30 days of completion of the visit. If corrective actions need to be taken by the manufacturer, WHO will postpone its final recommendations to the concerned United Nations agencies until such corrections are implemented and verified by WHO. If the company does not comply with the agreed deadlines, the prequalification process may be terminated.

3.6 Report and outcome of the assessment

When required, the final decision on the acceptability of the product for supply to United Nations agencies may be taken in consultation with an ad hoc committee on vaccine prequalification convened by WHO for this purpose.
Once WHO considers that the process is complete, and if the outcome is satisfactory, the Organization will send a letter to the United Nations agencies and to the GAVI Alliance in the case of Advance Market Commitment- (AMC-)\(^1\) eligible products advising on: (a) the compliance of the vaccine with both the WHO requirements and the specifications of the relevant United Nations agency; and (b) the role of the NRA in certifying this. This letter will be copied to the manufacturer, the NRA/NCL responsible for lot release, the relevant WHO regional and country offices, the management of WHO's Department of Immunization, Vaccines and Biologicals, and the approved VVM manufacturer.

For AMC-eligible products, WHO will send a report to the GAVI Alliance and the AMC's Independent Assessment Committee, providing the rationale for confirming or otherwise that the vaccine meets the target product profile.

The vaccine will be included in the WHO list of prequalified vaccines immediately after the letter is sent to the United Nations agencies. A page providing the basis for the acceptance of the prequalification of the specific vaccine will also be included in the list. The current list may be consulted on the WHO web site (4). In the event of disagreement between the manufacturer and WHO, a standard operating procedure for the handling of such disagreements will be followed, in order to discuss and resolve the issue.

The prequalified status of a vaccine is valid until a new reassessment is scheduled by WHO (see section 9). WHO reserves the right to revoke the prequalification status if fraud by the manufacturer becomes evident. For details on notification of changes or introduced variations, see section 7.

Communications, at any time, with the experts involved in a vaccine evaluation should be conducted through the WHO focal person in charge of the product.

### 4. Considerations for streamlining the prequalification procedure on the basis of enhanced assistance by NRAs

#### 4.1 Procedure for selecting eligible NRAs

Experience gained with the evaluation of influenza H1N1 (2009) pandemic vaccines showed that reliance on effective regulatory oversight by the responsible NRA has the potential to play a critical role in facilitating the prequalification process.

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\(^1\) An AMC is a legally-binding agreement for an amount of funds to subsidize the purchase, at a given price, of an as yet unavailable vaccine against a specific disease causing high morbidity and mortality in developing countries. The establishment of AMCs should encourage the development of future generations of vaccines and in particular accelerate the development and availability of priority new vaccines to developing countries.
procedure. It is considered that the experience in the context of pandemic influenza can be extrapolated to other vaccines.

The proposed procedure envisages enhanced reliance on the oversight carried out by the responsible NRA, when the authority exhibits a high level of performance of WHO’s six recommended regulatory functions and exercises full regulatory oversight of any given vaccine.

Full implementation of such an approach will require the development of a revised NRA assessment tool with additional performance indicators to supplement existing indicators. During the development and operational implementation of a revised tool able to distinguish levels of functionality (maturity levels), an interim selection process will be implemented with a limited number of NRAs with established regulatory capacity, in order to ensure standards for quality, safety and efficacy at least equivalent to those recommended by WHO (such as those published in the WHO Technical Report Series) (10).1

The interim process to be used for selection of NRAs will be:

- acceptance of NRAs that have provided enhanced support to WHO for pandemic H1N1 (2009) influenza vaccines;
- case-by-case analysis of feasibility for other potential NRAs, based on:
  - review of the established procedures and practices for marketing authorization/licensing of vaccines;
  - review and approval of variations/changes;
  - the extent of the ongoing regulatory oversight exercised for the vaccine of interest;
  - willingness of the agency to collaborate with WHO in the evaluation and ongoing regulatory oversight of the vaccine of interest.

Once the performance indicators have been developed and the NRA assessment tool is revised, thus allowing the establishment of functionality levels, a stepwise expansion to include additional authorities can be carried out.

4.2 Streamlined procedure for vaccines with marketing authorization/licensing granted by eligible NRAs

As an alternative to the WHO vaccine prequalification procedure described in section 3, the streamlined option can be applied to vaccines that have been

1 And subsequent updates to reference 10 published after every meeting of the WHO Expert Committee on Biological Standardization.
licensed by selected NRAs that are eligible and willing to share regulatory information with WHO through a collaboration agreement.

WHO will explicitly request the assistance of the NRA responsible for the regulatory oversight of the candidate vaccine, and will engage in discussions for the establishment of a formal collaboration agreement that outlines the shared understanding of roles, responsibilities and commitments of each party. Provisions for confidentiality will be included.

The scope of this agreement can be determined by both parties and could include one or more of the following (each subject to agreement by the manufacturer):

- sharing of NRA reports relevant to product quality, and nonclinical and clinical evaluation;
- sharing of NRA/NCL test results (including the raw data);
- sharing of inspection reports.

Once the collaboration agreement is formally established, depending on its nature and scope, WHO may decide, on a product-by-product basis, to do one or more of the following:

- review the NRA assessment reports instead of reviewing the PSF;
- review NRA/NCL testing results and their trending, if applicable, instead of independently testing the final product characteristics;
- review the NRA inspection reports and supplement this with a short audit focused on aspects related to United Nations tender specifications, instead of conducting a full site audit.

4.2.1 **Review of NRA assessment reports instead of the PSF**

In this case, WHO recognizes the assessment of the marketing authorization/licence dossier performed by selected NRAs responsible for the regulatory oversight of the candidate vaccine, as the basis for the decision on prequalification. WHO will review the NRA assessment and inspection reports instead of reviewing the PSF, and may follow up on queries on the basis of the information provided by the NRA responsible for the marketing authorization/licensing of the vaccine submitted for prequalification. If there are questions related to issues not addressed in the NRA reports, WHO will contact the manufacturer directly and copy the NRA on such exchanges of additional information.

Typically, the responsible NRA does not focus its review either on aspects that are specific to the national immunization schedules of countries that receive the vaccines through the United Nations, or on the programme needs stated in United Nations specifications. These elements must be assessed by WHO, except
in the case of the EMA scientific opinion procedure (Article 58 of Regulation (EC) No. 726/2004).

In view of the above, a review by WHO of the following aspects would remain essential:

- mandatory and critical characteristics from the programmatic point of view;
- eligibility, when required, for the AMC through review of the proposed product characteristics against the target product profile criteria;
- confirmation that the vaccine meets WHO recommendations;
- stability data to ensure that the vaccine meets the needs of immunization programmes in developing countries (particularly those with weak cold-chain systems), and assignment of a VVM category;
- clinical data to ensure that the vaccine is suitable for the target population;
- recommended immunization schedules to ensure compatibility with those of national immunization programmes;
- suitability of samples, labels, inserts and packaging to meet the United Nations agencies’ tender requirements;
- packaging for international shipment and its validation.

The applicant must provide WHO with a copy of the file submitted to the NRA and relevant sections of the PSF to cover information required on the items listed above.

An NRA that does not require renewal of the licence on a regular basis should have an alternative mechanism in place to conduct ongoing monitoring of the quality, safety and efficacy of the vaccines over which it exercises regulatory oversight. Updated information on these vaccines should be conveyed to WHO by the NRA at defined intervals. This information may be used in the reassessment procedure.

4.2.2 Review of NRA testing results and their trend, if applicable, instead of independently testing the consistency of final product characteristics

Vaccines submitted for the initial evaluation for prequalification are categorized by WHO into one of the four categories described in Appendix 5. Vaccines that meet the criteria described under categories I to III in Appendix 5 may be evaluated by applying the streamlined procedure.
In this case, WHO will recognize the lot release testing performed by the selected NRA/NCL responsible for the regulatory oversight of the candidate vaccine. WHO will review the available information (e.g. testing results, raw data, trends if applicable, and control charts). On the basis of the information provided by the NRA/NCL responsible for the lot release and testing of the vaccine submitted for prequalification, WHO will consider whether additional independent testing by WHO-contracted laboratories is required, or whether the information supplied can be accepted by WHO for prequalification purposes.

When the NRA/NCL responsible for the regulatory oversight does not perform the critical tests, whether for novel or traditional vaccines, testing by WHO-contracted laboratories must be conducted before the prequalification is granted.

4.2.3 Review of NRA inspection reports supplemented with a short audit focused on aspects related to United Nations tender specifications instead of conducting a full site audit

This procedure is based on WHO’s recognition of the inspections conducted by the selected NRAs responsible for the regulatory oversight of the candidate vaccine. The WHO site audit – as part of the initial evaluation, follow-up to corrective actions taken by the manufacturer following WHO recommendations, or reassessment – will be replaced by a review of inspection reports from the responsible NRA and a short audit by WHO that will include verification of specific items relevant to United Nations tender specifications.

If the review of inspection reports conducted by the responsible NRA is considered sufficient to ensure that vaccine candidates (or those already being purchased) meet or continue to meet the WHO requirements and specific conditions required for purchase by United Nations agencies, this information can be accepted by WHO for prequalification purposes.

WHO will include, as part of the agreement with the relevant NRA, an exchange of information regarding results of national inspections, variations to the licence (or cancellations), rejection of lots, recalls and withdrawals, interruptions in production, AEFIIs reported, or other matters that could affect the normal supply of vaccine to United Nations agencies.

4.2.4 Other considerations

The implementation of the streamlined prequalification procedure described above requires an eligible authority and the willingness of this authority to engage in a collaborative effort. Special attention should be given to authorities from countries where English is not the mother tongue. In such cases, engagement in this exercise would imply additional workload for the NRA in making its reports
available in English. Specificities of the collaboration (nature and extent) should be defined on a case-by-case basis and should be reflected in the agreement.

Vaccines that are produced for export-only purposes require special consideration and are not eligible for evaluation through the streamlined procedure described in section 4.2. In these cases, the report of the assessment is performed in accordance with the standard prequalification procedure (see section 3).

4.3 Vaccines with positive scientific opinion issued by the EMA

WHO is involved at different stages in the process of evaluation of vaccines by the EMA/CHMP under Article 58 (Regulation EC No. 726/2004). In this context, the EMA/CHMP issues a scientific opinion based on evidence of quality, safety and efficacy and taking into consideration the benefit–risk assessment for the intended population, which is consistent with WHO’s focus on developing countries.

All vaccine applications submitted for evaluation under Article 58, and intended for immediate prequalification after a positive scientific opinion, will be assessed through a streamlined procedure (see Appendix 6), in such a way that the time elapsed between the positive scientific opinion and prequalification will be minimized.

5. Special considerations for fast-track procedure

The implementation of a fast-track procedure may be required in special circumstances. This procedure is applicable to licensed vaccines (marketing authorization available) that are part of routine immunization programmes, or those that are used only in an emergency response; it is not applicable in the case of novel vaccines not yet introduced or recently introduced into routine immunization programmes.

In agreement with United Nations purchasing agencies or other partners, the fast-track procedure can be considered in the following situations:

- an acute shortage\(^1\) of a vaccine that puts at risk the global supply of routine immunization programmes and/or an eradication effort;
- an emergency situation (i.e. an outbreak or epidemic of a disease for which no prequalified vaccine is available, or where availability is insufficient and an additional source of the same vaccine is required);

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\(^1\) As agreed with United Nations purchasing agencies and other partners.
exceptional situations such as:

- declaration of a pandemic of a disease for which production capacity needs to be established;
- need for alternatives to existing vaccines to be used during an eradication effort.

Any of the above exceptional situations may lead to acceptance of vaccines for evaluation in parallel to submission to the NRA for marketing authorization purposes upon:

- special request from the manufacturer; and
- endorsement by senior management of WHO.

In cases where the fast-track procedure is followed, the established deadlines for submission of PSFs do not apply. In addition, the site audit will take place in parallel with quality control tests of samples while the results of tests are pending.

There should be maximum flexibility in this process. For example, review of the dossier and testing of samples will be concomitantly performed and the site audit will be conducted as soon as the dossier review is completed. As in the streamlined approach described under section 4, consideration should be given to review of information provided by the relevant regulatory authority with the manufacturer’s permission (including inspection reports), and to the results of tests performed by the relevant NRA/NCL to facilitate the evaluation process.

6. Special considerations for accepting submissions of vaccines manufactured at multiple sites or in different countries

It is a precondition of any submission of vaccines for prequalification evaluation that the NRA responsible for the regulatory oversight of the product must be assessed by a WHO team with respect to its compliance with the six critical functions identified by WHO. The functionality status of the NRA also needs to be sustained over time.

Owing to the increasing diversity and complexity of the vaccines that can be manufactured at multiple sites, including different countries, WHO has to ensure that the regulatory oversight is fully exercised and that responsibilities are clearly defined at all stages of production by the relevant functional NRAs. Certain criteria will be applied, as described here.
The assessment evaluation will be product-specific, as for vaccines produced by one company at a single site or in one country.

If a company formulates and/or fills from bulks (company A) purchased from different sources (companies B and C) each of these final products is considered as a unique product and will be prequalified separately.

If the formulation process used by the manufacturer of finished product of a vaccine (company A) is different from that used by the manufacturer of the vaccine from seed (company B) (e.g. different formulation procedures, different stabilizers, different adjuvants, different preservatives and/or different excipients), these vaccines will be considered unique products and may require preclinical and clinical evaluation.

Evidence will be required by WHO that the manufacturer of the finished product has authorization from the vaccine manufacturer producing the bulk to export the final product. In a case where purchased bulk antigen A is used for combination with antigens B and C from other sources, proper authorization by the bulk producer of antigen A for combination (and possible limitations on distribution of the combination vaccines) is required.

There must be a long-term contract between manufacturers, although a minimum of two years can be acceptable if justified. The technical terms and the duration of the contract must be submitted to WHO for review as part of the assessment procedure and, whenever necessary, additional information can be requested from the manufacturers.

For a manufacturer with subsidiaries in different parts of the world that perform different manufacturing steps, and if the bulk is not part of a licensed final product in the country of manufacture, the NRA of the country where the finished product is manufactured will need to exercise full regulatory oversight of the product. This means that this NRA is responsible for technical, nonclinical and clinical review, and for regulatory inspections of the facilities in each country performing manufacturing operations. This NRA would also grant the marketing authorization, perform lot release, including testing as necessary, as well as post-marketing surveillance.

For finished product manufacturers of OPV vaccines to be eligible for the prequalification process, as an exception the bulk material must have been evaluated as part of a vaccine already prequalified by WHO for the United Nations market.

In cases where the vaccine manufacture is conducted in more than one country, which may not be fully covered by the above provisions, the following aspects should be considered in order to ensure the ongoing regulatory oversight of vaccines:
Responsibility for overseeing manufacturing of different production steps should be shared between the relevant NRAs (functionality being a condition), with relevant agreements in place, and marketing authorization/licensing and release should be under the responsibility of the NRA of the country where the vaccine is distributed; consideration may be given to use of Article 58 of Regulation (EC) No. 726/2004 if the applicant is based in the European Economic Area (EEA), or has a contact point within the EEA; use of a production site in a country in which the NRA has not been assessed as functional requires that the NRA in the country of manufacture of the final product takes full responsibility for the oversight of the product. If this does not apply and/or Article 58 of Regulation (EC) No. 726/2004 cannot be used for any reason, this production site becomes unacceptable for a product to be evaluated for purchase through United Nations agencies.

The use of a totally unrelated (third-party) NRA for the oversight of the product (outside of the option of Article 58 of Regulation (EC) No. 726/2004) would not normally be acceptable. However, if an agreement between NRAs is established for a specific product, giving the third-party authority full regulatory responsibility that includes lot release for United Nations purposes, regular inspections, monitoring of variations, and post-marketing surveillance, then WHO would review the terms of agreement between the NRAs and make a case-by-case decision on acceptability.

WHO encourages early discussions with manufacturers and their respective NRAs if they plan to embark on a project involving multiple sites or countries in the production process, in order to discuss the proposed scheme and allocation of responsibilities to the NRAs.

7. Obligations after prequalification is granted

All lots of prequalified vaccine shipped in response to orders placed by a United Nations agency must have been released by the NRA in advance of shipping. Copies of the lot release certificates will be kept by the manufacturer and sent, on request, to the United Nations agencies or to the Coordinator, Quality, Safety and Standards, Department of Essential Medicines and Health Products, WHO, Geneva. In addition, a suitable number of samples (defined during the assessment process) of each vaccine lot supplied to the United Nations agencies will be retained by the manufacturer, in order to be made available to WHO on request, for testing.
The manufacturer must inform WHO of all changes/variations that must be notified or submitted to the NRA regarding the formulation, presentation, methods of manufacture or quality control, specifications, facilities, or any other aspects that might (a) result in a change of safety and/or efficacy of the vaccine; or (b) change the basis of the regulatory approval of the NRA.

If the regulations of the manufacturing country do not require approval by the NRA of changes/variations that fall under (a) and (b) above, WHO must be informed of the proposed changes before these are implemented on products supplied to United Nations agencies.

When WHO relies on the oversight of changes/variations by the responsible NRA, an annual summary of changes/variations (see section 8) will be sufficient.

When such reliance is not established, changes/variations that fall under (a) and (b) above must be accepted by WHO before United Nations supply. All other changes/variations can be reported to WHO on an annual basis, as detailed in section 8.

If the labelling specifications are changed or model inserts are updated as part of United Nations tender requirements, manufacturers must comply with the revised United Nations tender specifications. The updated versions of labels and package inserts must be reviewed by WHO before implementation.

WHO reserves the right to take appropriate measures, including “suspension of supply, initiating a reassessment or withdrawal from the list” in the case of noncompliance with post-prequalification commitments and/or in the case of misconduct.

**8. Annual reporting**

The following information should be provided in an annual report for each prequalified vaccine.

A. The manufacturer should provide a summary of changes/variations to the product(s) that have been implemented since the previous annual report (or, for the first annual report on a product, since initial prequalification). Table A6.1 is provided as guidance.
Table A6.1
Summary of changes/variations to the product report

<table>
<thead>
<tr>
<th>Description of variation</th>
<th>PSF chapter/section</th>
<th>CTD cross-reference (where appropriate)</th>
<th>Responsible NRA(^a)</th>
<th>WHO prior acceptance date (where required); or WHO notification date (as applicable)(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Prior approval date</td>
<td>Date of acknowledgement of notifiable change(^b)</td>
</tr>
</tbody>
</table>

\(^a\) For the columns under the heading “Responsible NRA”, one of the three choices will be relevant and the manufacturer should provide the requested information in the relevant column.

\(^b\) Provide the date of the NRA letter acknowledging the notification, or indicate if the NRA has not responded and hence give the date the change was implemented under national law.

\(^c\) See “Points to consider” in reference 11.
B. The manufacturer should provide testing results from the ongoing stability programme since the previous annual report (or, for the first annual report on a product, since initial prequalification).

- Production and distribution data should include a summary table showing the quantity of batches and doses of finished product distributed since the previous annual report. The table should include product used domestically and product exported. The batches supplied to United Nations agencies should be indicated. If more than one presentation is manufactured, these should be listed separately.

C. The manufacturer should provide details of GMP inspections (in which the prequalified product was within the scope of inspection) performed since the previous annual report.

D. A summary update on implementation of post-prequalification commitments should be provided by the manufacturer if these are indicated in the approval letter or reassessment report. These may be, for instance:

- reports of serious adverse events following immunization;
- reports of quality complaints and/or recalls from the field for batches of the prequalified vaccine;
- notification of any problem/constraint in production or quality control that might affect the international supply of this vaccine, both in terms of volume and/or lead times.

E. The periodic safety update report should be provided (electronic data only).

Following review of the annual report, WHO may request supporting data. The deadline for submission of the first annual report should be one year after the date of prequalification, with subsequent submissions each year on the same date. The manufacturer may provide the latest annual report submitted to the NRA provided that this contains the relevant information. The established deadlines for submission of PSFs will apply (31 January, 31 May and 30 September).

9. Reassessments

Prequalification status is maintained until action is taken by WHO to revoke it. However, periodic reassessment by WHO is required. The frequency, scope and need for reassessment will be based on quality risk management principles.
The following aspects will be taken into consideration by WHO:

- stringency of oversight exercised by the responsible NRA;
- prior experience with the manufacturer and the specific product;
- variations to the product indicated in annual reports since the previous assessment;
- interruptions to production and/or supply to United Nations agencies;
- reported quality complaints and AEFIs;
- any failure to meet the WHO recommendations and/or the specifications of the offer to bid;
- results from targeted testing of batches supplied to United Nations agencies.

The above list is indicative but not exhaustive.

A letter to the manufacturer requesting submission of an updated PSF for reassessment should be made 6–12 months prior to the time of the proposed assessment. Unless a paper copy is requested by WHO, the updated PSF should be in electronic form only. The updated PSF should contain a change control section, which indicates the sections that have been changed from the previously submitted PSF.

Items indicated in the change control section will be compared with summary tables of variations that have been submitted annually. The changed sections will also be compared to the file that was submitted initially. Only sections indicated as changed will be evaluated. Changes made that are not indicated in the change control section will not be considered as approved.

Testing of samples at reassessment is required only when there is insufficient evidence of continued compliance with specifications of the WHO annual targeted testing programme of batches supplied to United Nations agencies.

Consideration of the need for and scope of a site audit at the time of reassessment will take into account the demonstrated history of regulatory inspection of the facility by the NRA (including reports of GMP inspections by the NRA).

If, as a result of the reassessment, it is found that a vaccine no longer complies with the WHO-recommended standards, the vaccine will be removed from the list. Failure of a manufacturer to participate in the reassessment procedure will also lead to removal from the list.

10. Monitoring continued compliance with specifications through targeted testing

Samples of lots supplied through United Nations agencies will be selected at least once a year for testing of final product characteristics by WHO-contracted
laboratories. An appropriate number of samples (between 25 and 200, depending on the vaccine type and presentation offered) of three to five lots selected by WHO from a list of products supplied to United Nations agencies will be requested from the manufacturer. The manufacturer will provide lot summary protocols and the NRA/NCL release certificate as appropriate, for review. Manufacturers should commit to keeping an adequate number of retention samples for this testing programme.

Manufacturers will, in any case, be contacted for follow-up actions if they fail to meet specifications.

In the event of failure to meet the established criteria, WHO will investigate the problem and provide the United Nations agency with written information, copied to the manufacturer and the NRA, on the actions that need to be taken.

11. Monitoring vaccine quality complaints or AEFIs from the field

11.1 Vaccine quality complaints

In the case of vaccine quality complaints, WHO will conduct an investigation and may perform independent testing after review of the relevant documentation, including review of the temperature-monitoring devices, the testing results and related data.

In the case of complaints from NCLs in the receiving countries, the testing results and related documentation (i.e. validation reports, standard operating procedures and control charts) from the laboratory that puts forward the complaint are needed for WHO review before arbitration testing is commissioned.

11.2 AEFIs

In the case of serious AEFIs, or whenever WHO considers necessary, the Organization will conduct an investigation according to established procedure (12). The review of the batch records by the manufacturer and the NRA exercising the regulatory oversight of the vaccine allows for detection of any potential deviation during the manufacturing process that may impact on the quality of the vaccine.

The targeted testing programme, performed by WHO on a continuous basis, supports the continued compliance of the vaccine with the established quality specifications. In addition, testing results gathered during the lot release process by the NRA/NCL are requested from the NRA/NCL exercising
the regulatory oversight of the vaccine when AEFIs are investigated. Further testing would be resource-intensive and may not yield useful data. Therefore, the testing of a vaccine lot/batch will be recommended only if the clinical and/or epidemiological information about the AEFI case(s) indicates a potential vaccine quality problem and after review of the relevant manufacturing and control documentation. The investigation of AEFI cases will indicate whether testing is required and, if so, which type of test(s).

Depending on the tests to be performed, the number of unopened containers required for testing (sampled from the field and from the manufacturer) needs to be calculated so that the sample is representative and allows definitive conclusions to be drawn about the relevant lot. In the event that testing is needed, WHO will contact one of the WHO-contracted laboratories that can perform the test and subsequently inform the national authorities of the number of vaccine vials to be sent to WHO, as well as of other logistical arrangements.

12. Recommendations for action in cases of non-compliance

In the event of situations as described in sections 10 and 11 above, and depending on the nature of the non-compliance, WHO may recommend one or both of the following:

- the manufacturers’ lots of vaccines should be more closely monitored through additional testing, visits to the manufacturing facilities together with the NRA responsible for the regulatory oversight of the product, and/or review by WHO of the corrective/preventive actions during a probationary period;

- purchase of the vaccine by United Nations agencies should be suspended pending investigation and resolution of the problem.

Failures relating to gaps in the manufacturing and/or quality system of the manufacturer may require a complete reassessment of the vaccine. WHO will inform the NRA responsible for the regulatory oversight about problems in the field or failure to meet established criteria.

13. Handling out-of-specification/inconsistent results between laboratories

Owing to the increased complexity of the vaccines and new combinations currently available or in the pipeline for prequalification, challenges may be posed by the diversity of the methods applied for the quality control of vaccines,
as well as by the evaluation of results obtained through independent testing of such vaccines by WHO-contracted laboratories.

In the case of inconsistent results from two WHO-contracted laboratories, WHO may require testing of the vaccine by a third laboratory.

WHO may convene an ad hoc committee of experts to assess the combined results and make a recommendation to the Organization. Representatives from the WHO laboratories may take part in this committee. The recommendation from the committee will be considered as final by the prequalification secretariat.

14. Costs

The cost of the activities required to assess the acceptability, in principle, of candidate vaccines for United Nations agency purchase is covered by the manufacturers. It involves a screening fee and an evaluation fee. Both are paid after the screening of the PSF has been completed. If the screening process is not satisfactory, the manufacturer will be charged only the screening fee.

The expenses related to the site audit are charged on a cost-recovery basis. The evaluation of a vaccine commences only after payment of the fee and receipt by WHO of the PSF.

The cost of activities required to keep the WHO list updated, or maintenance fee (i.e. review of annual reports, reassessments, handling of complaints and resolution of out-of-specification results), is charged to the manufacturers, as an annual fee at the beginning of each calendar year. The expenses related to reassessment site audits are charged on a cost-recovery basis. The reassessment process will not be initiated until the corresponding fee is paid to WHO. Failure to pay could ultimately lead to withdrawal of the vaccines from the list.

In all cases where follow-up site audits and other additional activities and resources are required for special reasons (e.g. failure to meet the criteria), these will be charged separately on a cost-recovery basis. Fees will be updated regularly.

Fees (screening, initial evaluation of candidate vaccines, and annual maintenance) are kept on a separate list available on the WHO web site, along with other information and guidance documents for vaccine manufacturers (11).

15. Confidentiality

Information to which WHO requires access for the purpose of assessing or reassessing the acceptability, in principle, of a vaccine for purchase by United Nations agencies may include confidential information. However, if, in the opinion of the manufacturer, any information submitted to WHO and its expert team members in the course of the (re)assessment procedure includes confidential
information, the manufacturer must advise WHO of this in writing prior to, or at the same time as, the disclosure, duly identifying the confidential information in question. Notwithstanding the above, WHO and its expert team members will treat all information submitted to them either as written documents or during site audits as confidential, in accordance with the terms set out here.

WHO will treat any information contained in the PSF (see Appendix 1) and information disclosed during site audits as confidential and proprietary to the manufacturer. In this connection, WHO will take all reasonable measures to ensure that (a) the confidential information is not used for any other purpose than the (re)assessment procedure described in this document; and (b) the confidential information is not disclosed or provided to any person who is not bound by similar obligations of confidentiality and non-use.

WHO and/or its expert team members will not, however, be bound by any obligations of confidentiality and non-use to the extent they are clearly able to demonstrate that any part of the confidential information:

- was known to them prior to any disclosure by the manufacturer; or
- was in the public domain at the time of disclosure by the manufacturer; or
- has become part of the public domain through no fault of WHO and/or any of its expert team members; or
- has become available to WHO and/or any of its expert team members from a third party, not in breach of any legal obligations of confidentiality to the manufacturer.

In connection with the above, WHO requires all experts to sign the confidentiality agreement attached as Appendix 3, prior to taking up their responsibilities for WHO.

16. Conflict of interest

The team of experts selected for a specific evaluation process includes experts in the fields of production, quality control/quality assurance, quality system, clinical evaluation and GMP. These experts are selected by WHO and act as WHO temporary advisers or consultants. Prior to formalizing arrangements with such experts, WHO will also require them to complete the WHO declaration of interests, which is attached as Appendix 4. In addition, the confidentiality agreement referred to in section 15 contains a conflict-of-interest undertaking, pursuant to which the experts agree to discharge their functions exclusively as advisers to WHO. They also confirm that they have no financial interest and/or other relationship with a party that:
may have a vested commercial interest in obtaining access to any confidential information disclosed by the manufacturer in the course of the (re)assessment procedure described in this document; and/or

may have a vested interest in the outcome of the (re)assessment procedure, including, but not limited to, parties such as the manufacturer of the vaccine(s) that is (are) being assessed or manufacturers of competing vaccines.

WHO will advise the manufacturer in advance of the composition of the evaluation team and will provide the curricula vitae of the experts. The manufacturer will then have the opportunity to express possible concerns regarding any of the expert team members. If such concerns cannot be resolved in consultation with WHO, the manufacturer may reject an expert team member within, at the latest, 15 days of receipt of the proposed team composition.

Authors

The proposals for the revision of the Procedure for assessing the acceptability, in principle, of vaccines for purchase by United Nations agencies were prepared by:

Working group 1: Programmatic suitability of vaccines for WHO prequalification (PSPQ) membership – Dr R. Biellik, Consultant, La Rippe, Switzerland; Dr M. Cortes, Pan American Health Organization, Washington, DC, United States of America (USA); Dr N. Dellepiane, World Health Organization, Geneva, Switzerland; Dr R. Eggers, World Health Organization, Geneva, Switzerland; Dr M. Landaverde, Pan American Health Organization, Washington, DC, USA; Dr C. Nelson, Independent (working group chair), Pennsylvania, USA; Dr A. Ottosen, UNICEF Supply Division, Copenhagen, Denmark; Dr M. Pereira, Pan American Health Organization, Washington, DC, USA; Dr P. Pharmaphornpilas, Ministry of Public Health, Bangkok, Thailand; Ms E. Uramis, World Health Organization, Geneva, Switzerland; Working group 2: Comparison of prequalification programmes membership – Dr D. Meek, World Health Organization, Geneva, Switzerland; Dr A. Sands, World Health Organization, Geneva, Switzerland, Dr M. Zaim, World Health Organization, Geneva, Switzerland; Dr A. Van Zyl, World Health Organization, Geneva, Switzerland; Working group 3: Revised approaches to testing final product characteristics membership – Mrs T. Jivapaisarnpong, Ministry of Public Health, Bangkok, Thailand; Ms C. Rodriguez, World Health Organization, Geneva, Switzerland; Dr U. Rosskopf, World Health Organization, Geneva, Switzerland; Dr L. Tesolin, Scientific Institute of Public Health, Brussels, Belgium; Dr W. Vergeer, National Control Laboratory, Bloemfontein, South Africa; Dr G. Waeterloos, Scientific
Institute of Public Health, Brussels, Belgium; Working group 4: Streamlining the prequalification procedures for products with EMA/CHMP positive scientific opinion membership – D. Cockburn, European Medicines Agency, London, England; Dr E. Cooke, European Medicines Agency, London, England; Dr L. Chocarro, World Health Organization, Geneva, Switzerland; Dr M.-H. Pinheiro, European Medicines Agency, London, England; Dr L. Rago, World Health Organization, Geneva, Switzerland; Ms C. Rodriguez, World Health Organization, Geneva, Switzerland; A. Spina, European Medicines Agency, London, England; Working group 5: WHO assessment of vaccines regulatory system: proposal for establishment of maturity level concept membership – Dr N. Baylor, United States Food and Drug Administration, Rockville, MD, USA; Mr L. Belgharbi, World Health Organization, Geneva, Switzerland (working group chair); Dr P.H. Bertoye, Agence Française de Sécurité Sanitaire de Produits de Santé, Paris, France; Dr F. Fathalla, Egyptian National Control Laboratory, Cairo, Egypt; Dr S. Guichard, WHO Regional Office for South-East Asia, Delhi, India; Mrs T. Jivapaisarnpong, Ministry of Public of Health, Bangkok, Thailand; Mr Y. Ndao, Ministry of Finance and Economy, Dakar, Senegal; Dr J. Peña, Pan American Health Organization, Washington, DC, USA; Dr F. Reigel, Consultant, Basel area, Switzerland; Dr C. Rolls, Therapeutic Goods Administration, Woden, ACT, Australia; Dr C. Sánchez, Centro para el Control Estatal de la Calidad de los Medicamentos, Havana, Cuba; Dr S. Singh, Central Drug Standard Control Organization, New Delhi, India; Dr L. Slamet, National Agency of Food and Drug Control, Jakarta, Indonesia; Dr H. Wali, Egyptian National Control Laboratory, Cairo, Egypt; Working group 6: Requirements for product summary file submitted for prequalification. Initial evaluation and reassessment and requirements for annual report for prequalified vaccines membership – Dr N. Dellepiane, World Health Organization, Geneva, Switzerland; Dr J. Fournier-Caruana, World Health Organization, Geneva, Switzerland; Dr S. Lambert, World Health Organization, Geneva, Switzerland; Dr D. Meek (working group chair), World Health Organization, Geneva, Switzerland; Dr S. Nishioka, World Health Organization, Geneva, Switzerland; Ms C. Rodriguez, World Health Organization, Geneva, Switzerland; Dr U. Rosskopf, World Health Organization, Geneva, Switzerland; Working group 7: Streamlining the prequalification procedure: consideration of a risk-based approach membership – Dr J.W. Blair, United States Food and Drug Administration, Rockville, MD, USA; Dr N. Dellepiane, World Health Organization, Geneva, Switzerland; Dr R. Dobbelaer, Consultant, Lokeren, Belgium; Mr R.D. Morales, Centro Control Estatal de la Calidad de los Medicamentos, Havana, Cuba; Dr J. Southern, Temporary Adviser, Pretoria, South Africa; Ms E. Uramis, World Health Organization, Geneva, Switzerland; Dr H. van de Donk, Temporary Adviser, Den Haag, the Netherlands; Working group 8: Regulatory oversight of vaccines manufactured in multiple sites/countries membership – Dr N. Dellepiane, World
Health Organization, Geneva, Switzerland; Dr J. Fournier-Caruana (working group chair), World Health Organization, Geneva, Switzerland; Dr S. Lambert, World Health Organization, Geneva, Switzerland; Dr D. Meek, World Health Organization, Geneva, Switzerland; Dr S. Nishioka, World Health Organization, Geneva, Switzerland; Ms C. Rodriguez, World Health Organization, Geneva, Switzerland; Dr U. Rosskopf, World Health Organization, Geneva, Switzerland.

The proposals were discussed at the Informal consultation with the ad hoc committee on vaccines prequalification for the revision of the procedure for assessing the acceptability, in principle, of vaccines for purchase by United Nations agencies and recommendations were received from the ad hoc committee members – Dr J.W. Blair, United States Food and Drug Administration, Rockville, MD, USA; Ms L.G. Castanheira, ANVISA, Brasília, Brazil; Dr P. Chagnaud, Agence Française de Sécurite Sanitaire de Produits de Santé, Paris, France; Ms X. Chen, State Food and Drug Administration, Beijing, China; Dr E. Cooke, European Medicines Agency, London, England; Dr R. Dobbelaer, Lokeren, Belgium; Dr M. Eisenhawer, Swiss Agency for Therapeutic Products Inspectorates, Berne, Switzerland; Dr I. Feavers, National Institute for Biological Standards and Control, Potters Bar, England; Dr M. Ferguson, Consultant, Norfolk, England; Mrs T. Jivapaisarnpong, Ministry of Public Health, Bangkok, Thailand; Dr J. Joung, Korea Food and Drug Administration, Seoul, Republic of Korea; Dr K. Midthun, Center for Biologics Evaluation and Research, Rockville, MD, USA; Mr R.D. Morales, Centro Control Estatal de la Calidad de los Medicamentos, Havana, Cuba; Dr R. Nibbeling, National Institute of Public Health and Environment Protection, Bilthoven, the Netherlands; Dr M.-H. Pinheiro, European Medicines Agency, London, England; Professor H. Rees, University of the Witwatersrand, Johannesburg, South Africa; Dr C. Rolls, Therapeutic Goods Administration, Woden, ACT, Australia; Dr L. Slamet, National Agency of Food and Drug Control, Jakarta, Indonesia; Dr V.G. Somani, Ministry of Health and Central Drugs Standard Control Organisation, New Delhi, India; Dr J. Southern, Temporary Adviser, Pretoria, South Africa; Dr J-M. Spieser, European Pharmacopoeia Commission Secretariat, Strasbourg, France; Dr L. Tesolin, Scientific Institute of Public Health, Brussels, Belgium; Dr W. Vergeer, National Control Laboratory, Bloemfontein, South Africa; and other meeting participants.

The first draft of the revised procedure was prepared by the drafting group: Dr L. Chocarro, World Health Organization, Geneva, Switzerland; Dr N. Dellepiane, World Health Organization, Geneva, Switzerland; Dr D. Meek, World Health Organization, Geneva, Switzerland; Dr S. Nishioka, World Health Organization, Geneva, Switzerland; Ms C. Rodriguez, World Health Organization, Geneva, Switzerland; Dr U. Rosskopf, World Health Organization, Geneva, Switzerland; Ms E. Uramis, World Health Organization, Geneva, Switzerland;
and Dr D. Wood, World Health Organization, Geneva, Switzerland, taking into account the recommendations from the ad hoc committee members and posted on the WHO biologicals web site for public consultation.

On the basis of comments received from regulators, vaccine manufacturers and other experts, document WHO/BS/10.2155 was prepared by the drafting group and posted on the WHO biologicals web site for public consultation.

This final document was prepared by Ms E. Uramis and Dr N. Dellepiane on the basis of comments received from regulators, vaccine manufacturers, other experts and members and participants at the meeting of the Expert Committee on Biological Standardization in 2010.

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– Dr J. Fournier-Caruana, World Health Organization, Geneva, Switzerland;  
– Dr S. Lambert, World Health Organization, Geneva, Switzerland;  
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– Dr J. McEwen, Therapeutic Goods Administration, Woden, ACT, Australia;  
– Dr J. Milstien, University of Maryland, Baltimore, MD, USA;  
– Mr R.D. Morales, Centro Control Estatal de la Calidad de los Medicamentos, Havana, Cuba;  
– Dr C. Nelson, Consultant, PA, USA;  
**Second draft**  
– Agence Française de Sécurité Sanitaire de Produits de Santé, Lyons, France;  
– BioFarma, West Java, Indonesia;  
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– Japan BCG Laboratory, Tokyo, Japan;  
– Novartis, Siena, Italy;  
– Panacea Biotec Limited, New
References


Appendix 1

The PSF

The PSF is a summary dossier containing current information on the product to be supplied to United Nations agencies. It presents information on the product composition, manufacturing procedure, testing, stability, labelling, clinical experience and available post-marketing safety information.

For initial product assessments, a PSF shall be submitted for each vaccine to be assessed. For combination vaccines, information shall be submitted on each of the component vaccines and on the combination itself. If a combination vaccine is being evaluated and the monovalent versions of the antigens contained in the combination are also being evaluated, the information provided for the monovalent vaccines (up to concentrated bulk) can be used for the assessment of the combinations or, conversely, the information on each antigen provided in the PSF of the combination vaccine can be used to assess the monovalent vaccines (up to concentrated bulk level).

The PSF is expected to contain the following elements.

Chapter 1: General information

1.1 Provide brief information on the company (including name and address of the site, telephone, fax and 24-hour telephone numbers, and the principal contacts of the company) and its relation to other sites where steps of the process or testing activities (for both the active biological components and diluent) may be conducted.

1.2 List pharmaceutical and non-pharmaceutical manufacturing activities carried out at the site, as licensed by the NRA. This information shall also be provided for contracted manufacturers.

1.3 Provide a short description of the site (size, location and immediate environment). List buildings on the site(s) or provide a site plan, identifying the manufacturing, control and storage activities in each building.

1.4 State the number of employees engaged in production, quality assurance, quality control, storage and distribution.

1.5 List outside scientific, analytical or other technical assistance in relation to manufacture and analysis, including equipment and/or other facility maintenance and validation. In the case of contract manufacturing and contract testing of part of the process, provide information on the way in which GMP compliance of the contract acceptor is assessed.
1.6 Give a short description of the quality management system of the company responsible for manufacture.

1.7 Give a short description of the internal audit system and the programme for qualifying suppliers of raw materials.

1.8 List the manufacturers supplying biological raw materials and adjuvants.

**Chapter 2: Personnel**

2.1 Provide an organizational chart showing the relationships between different areas, including quality assurance, production and quality control, with identification by name of key personnel (heads of production, quality assurance, quality control, warehousing and engineering).

2.2 Provide curricula vitae for heads of production, quality assurance and quality control, indicating educational and experience qualifications.

2.3 Outline arrangements for basic and continuing training and how records are maintained.

2.4 Describe requirements for personnel engaged in production, particularly relating to requirements for monitoring of health status (including immune status) of production personnel, and for outside contract service personnel entering the manufacturing areas.

**Chapter 3: Premises and equipment**

These will be examined in depth during the site audit. However, the following preliminary information must be submitted.

3.1 Provide simple, currently valid, floor plans and text descriptions of manufacturing and quality control areas. The floor plans should give an indication of scale, air flow and flows of materials, product, personnel and waste (architectural or engineering drawings are not required), room classification, and air handling unit identification by room.

3.2 Describe the nature of construction and finishes of manufacturing and quality control areas.

3.3 Describe ventilation systems in the manufacturing and quality control areas. More details should be given for critical areas with potential risks of airborne contamination (schematic drawings of the systems are desirable). Classification of the clean rooms used for the manufacture of sterile products should be included. A description of the environmental monitoring programme is required.
3.4 Provide information on special areas for the handling of highly toxic, hazardous and sensitizing materials.

3.5 Describe water systems (schematic drawings of the systems are desirable, showing storage tanks, loops, points of use and sampling points), including sanitation procedures and schedules. A description of quality control testing and schedules is required.

3.6 Describe the maintenance system (planned preventive maintenance programmes and recording system).

3.7 Complete a table (as in the example shown), briefly describing major production and control laboratory equipment used for the production of the vaccine (including diluent).

<table>
<thead>
<tr>
<th>Room ID</th>
<th>Major equipment in room</th>
<th>Clean room class</th>
</tr>
</thead>
</table>

3.8 For products where a separate facility is required (e.g. tetanus, bacille Calmette-Guérin vaccine [BCG]), describe how separation is achieved.

3.9 Describe qualification and validation procedures, including computerized recording and controller systems. A description of the validation master plan is required.

3.10 Provide a brief description of the procedures for cleaning manufacturing areas and equipment. For multipurpose areas, briefly describe the system for cleaning and testing between campaigns.

**Chapter 4: Vaccine composition, presentations and schedules**

4.1 State the composition of the product (including diluents).

4.2 Describe the presentations made available to United Nations agencies, including diluents (if applicable), combination products, forms, dose sizes, type of containers, VVM type used, and descriptions of application devices (e.g. autodisable syringes) to be delivered with the vaccine, if applicable.

4.3 Give the recommended schedule and route of administration.

4.4 For both the final product and diluent, provide samples of primary container, labels, boxes and package inserts to be used for United Nations agency supply (in English). French, Portuguese, Russian and Spanish versions need
to be made available before supply to United Nations agencies starts. Include the calculated volume per dose in cm$^3$ of the secondary packaging.

4.5 Include a sample of the lot summary protocol to be provided to United Nations agencies (using the WHO-recommended format).

Chapter 5: Production

5.1 Provide the following:

- the manufacturing formulae for the production of each antigen in the vaccine (i.e. fermenter or culture volumes for each bulk batch size, as applicable, and typical bulk volumes per production run);
- the batching formula for each batch size of final formulated bulk product;
- the approximate number of vials and doses for each fill size and presentation;
- the lot numbering system for intermediates and final products.

5.2 Provide a description of the manufacturing processes and the characterization of the product. This should include history of the master cell banks/virus seeds. Detailed flowcharts should be provided to indicate:

- each manufacturing step;
- the location (building/room) of each step, and transfers to other buildings/sites, if applicable;
- in-process and quality control tests performed on all intermediates and final products;
- identification of any processes or tests performed by contract manufacturers or testers;
- storage times and temperatures of intermediates.

For recombinant vaccines, a description of the construction and characterization of the recombinant vector, as well as the source of master cell bank/constructs, shall be provided. Include details of the manufacture and quality control of any adjuvant and diluents.

5.3 Describe the general policy for process validation. List the process-validation activities performed.

---

1 WHO recommendations or guidelines and United Nations agencies’ tender specifications must be met. For each specific test done, the international standard met should be identified.
5.4 Summarize arrangements for the handling of starting materials, packaging materials, bulk and finished products, including sampling, quarantine, release and storage.

5.5 Summarize arrangements for the handling of rejected materials and products, and procedures for their destruction.

Chapter 6: Quality control

6.1 Starting materials

6.1.1 List the control tests performed on raw materials, with appropriate characterization of starting materials, namely:

- list of raw materials meeting compendial specifications, indicating the pharmacopoeia;
- list of raw materials meeting in-house specifications, including the tests performed and specifications;
- list of biological starting materials (human or animal origin) with information on the requirements to avoid risk of transmissible spongiform encephalopathies and human diseases (HIV, hepatitis, etc.) in the final product;
- list of media with ingredients, tests performed and specifications.

6.1.2 List the control tests performed on labelling and packaging material(s), including primary and secondary packaging material.

6.1.3 Describe the qualification criteria for suppliers of raw material and relevant certificates.

6.2 Intermediate products (as appropriate)

6.2.1 List the routine tests performed and specifications for intermediates. Include copies of standard operating procedures for critical quality-control tests (uncontrolled copies or concise descriptions of the method and re-test criteria are acceptable).

6.2.2 List the assay validation activities performed.

6.3 Finished product (including diluent)

6.3.1 List the routine tests performed and specifications for the final product. Concise descriptions of the method and retest criteria are acceptable but full standard operating procedures in English should be made available on request.
6.3.2 List the assay validation activities performed.

6.3.3 List the final lots internally rejected in the previous 2 years and the reasons for rejection.

Chapter 7: Stability

Stability studies are expected to have been designed and conducted to meet WHO guidance (1).

7.1 Provide information on stability tests on intermediates, namely:

- information on containers for intermediate products;
- assigned shelf-life and storage conditions;
- quality control methods and specifications, and rationale for the choice of tests for determining stability;
- identification of the dates of manufacture of the lots, the lot numbers, the vial and dose size and the scale of production.

Results of quantitative assays must be expressed as a numerical value with the appropriate limits and not as “pass” or “fail”.

7.2 For each presentation, provide information on stability testing of the finished product, namely:

- assigned shelf-life and storage conditions;
- quality control methods and specifications, and rationale for the choice of tests for determining stability profile;
- identification of the dates of manufacture of the lots, the lot numbers, the vial and dose size and the scale of production.

Results of quantitative assays must be expressed as a numerical value with the appropriate limits and not as “pass” or “fail”.

In addition to data on final product stability at the recommended storage temperature, the accelerated stability data at elevated temperatures should be sufficient to justify the choice of VVM for use with the product (2).

7.3 Provide information on stability testing of diluents and reconstituted vaccine in the case of lyophilized vaccines.

7.4 Describe the policy for assigning the date of manufacture of each component, as well as the final product (e.g. combination vaccine) and diluents, as appropriate.
Chapter 8: Clinical experience

Note 1: Clinical studies are expected to have been designed and conducted to meet WHO and international GCP principles. Applicants should consult the following documents and any related updates in the WHO Technical Report Series:

- Guidelines on clinical evaluation of vaccines: regulatory expectations (3)
- WHO Guidelines on nonclinical evaluation of vaccines (4)
- Guidelines for good clinical practice (GCP) for trials on pharmaceutical products (5).

Other guidance documents are:

- Clinical considerations for evaluation of vaccines for prequalification (6)
- ICH guidelines (7).

Note 2: For vaccines whose licence was originally obtained many years before the application for WHO prequalification, it is possible that many or all of the clinical trials may not have been conducted or monitored to current international standards. For these vaccines, all sections should be completed but additional emphasis should be given to information provided in sections 8.2.1, 8.2.5, 8.3.1 and 8.3.2 in order to establish sufficiently a history of safe and effective use.

Note 3: In some cases, where the information received regarding the sections detailed below is not sufficient, is not clear enough or requires further scrutiny, WHO may request the applicant to submit the raw data.

8.1 The applicant should provide a tabulated summary of the clinical development programme in one or more tables, in which critical parameters that may have changed during the clinical development should be mentioned.

8.2 Clinical trials information

8.2.1 Overview of clinical trials sponsored by the applicant

The sponsor should provide a list of all clinical trials performed in all countries that are relevant to the application for WHO prequalification. These should include all studies sponsored by the applicant both before and at any time after initial licensure, whether or not submitted previously to the NRA(s) where the product is licensed. For each study on the list, the following information is required:

- final approved protocol, which should indicate the date of protocol approval by the ethics committee and the NRA;
- evidence of registration in a clinical trial registry that is included in the WHO International Clinical Trials Registry platform;
- indication of whether the study complied with GCP.

For each such study, in a tabulation or brief summary, the following information should be provided:

- the type of study;
- the rationale for its conduct;
- the location(s) of study sites;
- the dates of the study;
- the numbers and ages of subjects;
- a statement of final conclusions on safety and immunogenicity.

Copies of all publications and abstracts relating to these trials should accompany the submission in section 8.2.1.

In addition, the applicant should list any trials that are known to be currently ongoing, with a summary of details of the study plan and expected date of results.

8.2.2 Other studies with the applicant’s product

The applicant should make every effort to provide a list of all trials and, where applicable, observational studies relevant to the application that were not sponsored by the applicant but in which the product was evaluated. This list should be compiled from publications identified using an extensive literature search (details of which should be provided) and, in the case of co-licensure agreements, from any other company that holds a licence for or a right to market the same product.

8.2.3 Clinical summary

Provide a detailed summary and interpretation of the safety and efficacy data obtained from the pre-licensure clinical studies and all studies performed in the post-licensure period that support the current prescribing information. The summary should pay particular attention to any data that are relevant to the use of the product worldwide in WHO-recommended schedules (e.g. co-administration of other vaccines). In the absence of such data, the summary should provide a preclinical and/or clinical justification for the extrapolation of the existing data to the likely circumstances
of use after prequalification. This summary should complement, and not replace, the summary written by an independent clinical expert described in 8.2.5.

8.2.4 *Assessment reports*

Whenever possible, the applicant should provide the clinical sections of the NRA assessment reports from the country of origin and/or country where the vaccine is initially licensed. Assessment reports for both initial licensure and any subsequent variations to the licence for changes relevant to clinical data are requested.

8.2.5 *Clinical expert report*

Provide an independent clinical expert report on the clinical studies (evidence of expertise and independence should be provided). If the application for prequalification is based on the extrapolation of the existing clinical data to the likely circumstances of use after prequalification, and if the data are old or there is a doubt regarding the ethical or regulatory oversight of the trial, the report should discuss the degree of compliance with WHO GCP recommendations and current guidance regarding preclinical and clinical trials with vaccines.

8.2.6 *Preclinical studies sponsored by the applicant*

Provide a simple list of all preclinical studies that were sponsored by the applicant in support of use in clinical trials in humans, or for significant changes to manufacture or use. Include in the list any important conclusions. For preclinical studies performed after initial licensure, indicate the reasons for these studies. Any other particularly relevant reports regarding safety aspects, whether or not generated by the applicant, should be provided.

8.3 *Documentation of safety*

Safety data should be submitted both in the case of the initial application for prequalification evaluation and for reassessment purposes.

8.3.1 *Post-marketing pharmacovigilance*

Provide an outline of the post-marketing pharmacovigilance plan for the product.

8.3.2 *Initial evaluation of vaccines that have been on the market for a long time, or reassessment of already prequalified vaccines*

Provide an outline of the applicant’s procedures for the collection, onward notification and assessment of adverse events. Provide a
listing of all reported AEFIs for the vaccine in question in the last 5 years or since the last WHO reassessment. As far as is possible from the reports received, applicants should list the type of reaction, lot number, date and place of immunization, patients’ initials and age and, for immunization series, the dose number. A judgement of seriousness and whether or not the event was expected (in the light of the prescribing information) should be provided where this is possible from the information. An assessment of the relationship to the vaccine made by a clinician and, where relevant, by the applicant company or its independent clinical expert, should be included.

Whenever periodic safety updated reports (PSURs) are available, these shall be submitted. The PSURs should include information following the ICH format, from all geographical areas where the vaccine is used, or the absence of such information should be defended.

8.3.3 Recently licensed vaccines
In the case of vaccines that have recently been licensed, provide information on any ongoing phase IV studies or on any active monitoring of the safety profile that is taking place.

8.3.4 Documentation of serious adverse events
For serious adverse events reported in the last 5 years, or as long as the vaccine has been marketed (when shorter than 5 years), provide the fullest possible description of each case, including any information there may be on investigations, actions, patient treatment and outcome. This information should be provided as part of the PSUR.

Chapter 9: Production and distribution data
9.1 Provide information on the quantity of finished product distributed domestically and exported in the previous 3 years. List the different presentations separately, and indicate whether the list gives the numbers of vials or the numbers of doses distributed. When the product is a combination vaccine, information should also be provided on the history of distribution of component vaccine(s), when applicable.

9.2 Provide a list of countries where the product is licensed (marketing authorization) and supplied.

9.3 Summarize the arrangements and recording system for distribution, including the release process performed by the manufacturer and the NRA.
9.4 Summarize the packaging procedures for international shipments (including box sizes, packing volumes, etc.). Provide the validation protocols and reports of the shipping boxes used for United Nations supply. Recommendations provided in the most recent version of the WHO *Guidelines on the international packaging and shipping of vaccines* shall be followed (8).

9.5 Describe the arrangements for handling complaints and product recalls. Include description of the recall investigation system, procedures for corrective actions, and description of the regulatory requirements in case of recalls. Include provisions for informing WHO and the United Nations agencies.

9.6 Give the quantity of bulk vaccine destined for United Nations agencies that has been supplied to contract fillers/packagers for finalization (list individually).

**Chapter 10: Update on regulatory actions**

10.1 Provide a copy of regulatory documentation, namely:

- marketing authorizations for all formulations;
- information on refusals, withdrawals or suspensions, including those initiated by the manufacturer;
- the GMP certificate or equivalent.

10.2 Provide a list of lots rejected by the NRA, if applicable.

10.3 Describe restrictions on distribution or recalls, including manufacturer-initiated recalls.

10.4 Name clinical trial suspensions, including manufacturer-initiated suspensions.

10.5 Describe dosage or schedule modifications since the initial licensure was granted.

10.6 Provide information on changes in target populations or indications since the initial licensure was granted.

10.7 List inspections conducted by NRAs within the previous 2 years, including the scope of each inspection.

10.8 List inspections conducted by foreign authorities within the previous 2 years, including the scope of each inspection.
References


Appendix 2

Flowcharts of WHO prequalification for vaccines

Figure 1
Overall process

1. Submission of application letter
   - Is vaccine a priority for PQ? (Yes/No)
     - Yes: Acknowledge acceptance of intention to submit
     - No: Reject application

2. Product summary file submission
   - Screening
     - Is PSF complete? (Yes/No)
       - Yes: Mandatory PSPQ characteristics met?
         - Yes: Critical PSPQ characteristics met?
           - Yes: Forward for final assessment
           - No: No
         - No: No
       - No: Additional data request (Yes/No)
         - Yes: No
         - No: Are critical data missing? (Yes/No)
           - No: Forward for final assessment
           - Yes: No

Note: One month (or up to four months if PSPQ SC advice required)
This shape indicates a process delay point where action by the manufacturer is required. The time between request to the manufacturer for information and its supply is not part of the process time indicated.

PQ, prequalification; PSPQ, Programmatic suitability for prequalification; SC, Standing Committee.
Figure 2
Product summary file evaluation

Accepted PSF

Manufacturer accepts reviewers/pays fees

Data evaluation

Report

Additional data required? Yes

Are data satisfactory? No

Satisfactory completion of PSF evaluation process

No

Additional data required?

Are data satisfactory?

No

Refer to ad hoc vaccine PQ committee?

No

Process terminated

Yes

Refer to ad hoc vaccine PQ committee (see main process chart, Figure 1)

Three months

(for each round of data evaluation)

Request/supply of additional data

PQ, prequalification.

This shape indicates a process delay point where action by the manufacturer is required. The time between request to the manufacturer for information and its supply is not part of the process time indicated.
Figure 3
Testing process

This shape indicates a process delay point where action by the manufacturer is required. The time between request to the manufacturer for information and its supply is not part of the process time indicated.

PQ, prequalification.
Figure 4
Site audit

Satisfactory completion of PSF review

Schedule site audit

Site audit

Draft report (delivered at exit meeting)

Final report

Critical deficiencies found? Yes

Process terminated

No

Additional data required? Yes

Request/supply of additional data

Review of additional data satisfactory? Yes

Satisfactory completion of site audit process

No

Yes

Follow-up site visit required? Yes

No

Review by ad hoc committee (see main process chart, Figure 1)

Refer to ad hoc vaccine PQ committee?

Yes

No

PQ, prequalification.

This shape indicates a process delay point where action by the manufacturer is required. The time between request to the manufacturer for information and its supply is not part of the process time indicated.
Appendix 3

Confidentiality agreement

Provisions for team members participating in WHO missions to assess/reassess the acceptability, in principle, of vaccines for purchase by United Nations agencies

In the course of discharging your functions as an expert adviser under this Agreement, you will gain access to certain information that is proprietary to WHO or to the manufacturer(s) of the vaccine(s) that need(s) to be assessed for purchase by United Nations agencies. You undertake to treat such information (hereinafter referred to as “the Information”) as confidential and proprietary to WHO or the aforesaid manufacturer(s). In this connection, you agree to:

(1) not use the Information for any other purpose than discharging your obligations under this agreement; and
(2) not disclose or provide the Information to any person who is not bound by similar obligations of confidentiality and non-use as contained herein.

However, you will not be bound by any obligations of confidentiality and non-use to the extent that you are clearly able to demonstrate that any part of the Information:

(1) was known to you prior to any disclosure by WHO and/or the manufacturer(s); or
(2) was in the public domain at the time of disclosure by WHO and/or the manufacturer(s); or
(3) has become part of the public domain through no fault of your own; or
(4) has become available to you from a third party not in breach of any legal obligations of confidentiality to WHO and/or the manufacturer(s).

You also undertake not to communicate the deliberations and findings of the team(s) of experts in which you will participate, as well as any resulting recommendations and/or decisions of WHO, to any third party, except as explicitly agreed by WHO.

You will discharge your responsibilities hereunder exclusively in your capacity as an expert adviser to WHO. By signing this Agreement, you
furthermore confirm that you have no financial interest and/or other relationship with a party that:

1. may have a vested commercial interest in obtaining access to any part of the Information referred to above; and/or
2. may have a vested interest in the outcome of the assessment of the vaccine(s) in which you will participate, including but not limited to parties such as the manufacturer(s) of the vaccine(s) that is (are) being assessed, or manufacturers of competing vaccines.

In this regard, it should be noted that the manufacturer(s) of the vaccine(s) under evaluation have the right to object to your participation in the team(s) of experts that will evaluate (its) (their) vaccine(s). If such objection cannot be resolved in consultation with the manufacturer(s), WHO shall be entitled to terminate this Agreement or cancel part of the activities to be undertaken by you hereunder. The travel and per diem allowances payable to you under this Agreement will in such event be adjusted accordingly.

I hereby agree to the conditions and provisions contained in this document.

Signed: ____________________________________________
Name (typewritten): ______________________________________
Institute: _______________________________________________
Place: __________________________________________________
Date: ___________________________________________________
Appendix 4

Declaration of interests for WHO experts

WHO’s work on global health issues requires the assistance of external experts who may have interests related to their expertise. To ensure the highest integrity and public confidence in its activities, WHO requires that experts serving in an advisory role disclose any circumstances that could give rise to a potential conflict of interest related to the subject of the activity in which they will be involved.

All experts serving in an advisory role must disclose any circumstances that could represent a potential conflict of interest (i.e. any interest that may affect, or may reasonably be perceived to affect, the expert’s objectivity and independence). You must disclose on this Declaration of Interest (DOI) form any financial, professional or other interest relevant to the subject of the work or meeting that you have been asked to participate in or contribute towards and any interest that could be affected by the outcome of the meeting or work. You must also declare relevant interests of your immediate family members (see definition below) and, if you are aware of it, relevant interests of other parties with whom you have substantial common interests and that may be perceived as unduly influencing your judgement (e.g. employer, close professional associates, administrative unit or department).

Please complete this form and submit it to the WHO Secretariat, if possible at least 4 weeks, but no later than 2 weeks, before the meeting or work. You must also promptly inform the Secretariat if there is any change in this information prior to, or during the course of, the meeting or work. All experts must complete this form before participation in a WHO activity can be confirmed.

Answering “Yes” to a question on this form does not automatically disqualify you or limit your participation in a WHO activity. Your answers will be reviewed by the Secretariat to determine whether you have a conflict of interest relevant to the subject at hand. One of the outcomes listed in the next paragraph can occur depending on the circumstances (e.g. nature and magnitude of the interest, time frame and duration of the interest).

The Secretariat may conclude that no potential conflict exists or that the interest is irrelevant or insignificant. If, however, a declared interest is determined to be potentially or clearly significant, one or more of the following three measures for managing the conflict of interest may be applied. The Secretariat (i) allows full participation, with public disclosure of your interest; (ii) mandates partial exclusion (i.e. you will be excluded from that portion of the meeting or work related to the declared interest and from the corresponding decision-making
process); or (iii) mandates total exclusion (i.e. you will not be able to participate in any part of the meeting or work).

All potentially significant interests will be disclosed to the other participants at the start of the activity and you will be asked if there have been any changes. A summary of all declarations and actions taken to manage any declared interests will be published in resulting reports and work products. Furthermore, if the objectivity of the work or meeting in which you are involved is subsequently questioned, the contents of your DOI form may be made available by the Secretariat to persons outside WHO if the Director-General considers such disclosure to be in the best interest of the Organization, after consulting with you. Completing this DOI form means that you agree to these conditions.

If you are unable or unwilling to disclose the details of an interest that may pose a real or perceived conflict, you must disclose that a conflict of interest may exist and the Secretariat may decide that you be totally recused from the meeting or work concerned, after consulting with you.

Name: ____________________________________________
Institution: __________________________________________
E-mail: ____________________________________________

Date and title of meeting or work, including description of subject matter to be considered (if a number of substances or processes are to be evaluated, a list should be attached by the organizer of the activity):

__________________________________________________________________________

Please answer each of the questions below. If the answer to any of the questions is “yes”, briefly describe the circumstances on the last page of the form.

The term “you” refers to yourself and your immediate family members (i.e. spouse (or partner with whom you have a similar close personal relationship) and your children). “Commercial entity” includes any commercial business, an industry association, research institution or other enterprise whose funding is significantly derived from commercial sources with an interest related to the subject of the meeting or work. “Organization” includes a governmental, international or non-profit organization. “Meeting” includes a series or cycle of meetings.

EMPLOYMENT AND CONSULTING

Within the past 4 years, have you received remuneration from a commercial entity or other organization with an interest related to the subject of the meeting or work?
1a Employment  
1b Consulting, including service as a technical or other adviser  

RESEARCH SUPPORT  
Within the past 4 years, have you or has your research unit received support from a commercial entity or other organization with an interest related to the subject of the meeting or work?  

2a Research support, including grants, collaborations, sponsorships, and other funding  
2b Non-monetary support valued at more than US$ 1000 overall (include equipment, facilities, research assistants, paid travel to meetings, etc., support (including honoraria) for being on a speaker's bureau, giving speeches or training for a commercial entity or other organization with an interest related to the subject of the meeting or work?)  

INVESTMENT INTERESTS  
Do you have current investments (valued at more than US$ 10 000 overall) in a commercial entity with an interest related to the subject of the meeting or work? Please also include indirect investments such as a trust or holding company. You may exclude mutual funds, pension funds or similar investments that are broadly diversified and on which you exercise no control.  

3a Stocks, bonds, stock options, other securities (e.g. short sales)  
3b Commercial business interests (e.g. proprietorships, partnerships, joint ventures, board memberships, controlling interest in a company)  

INTELLECTUAL PROPERTY  
Do you have any intellectual property rights that might be enhanced or diminished by the outcome of the meeting or work?  

4a Patents, trademarks, or copyrights (including pending applications)  
4b Proprietary know-how in a substance, technology or process  

PUBLIC STATEMENTS AND POSITIONS (during the past 3 years)  
5a As part of a regulatory, legislative or judicial process, have you provided an expert opinion or testimony, related to the subject of the meeting or work, for a commercial entity or other organization?
5b Have you held an office or other position, paid or unpaid, where you represented interests or defended a position related to the subject of the meeting or work?  

Yes/No

ADDITIONAL INFORMATION

6a If not already disclosed above, have you worked for the competitor of a product that is the subject of the meeting or work, or will your participation in the meeting or work enable you to obtain access to a competitor’s confidential proprietary information, or create for you a personal, professional, financial or business competitive advantage?  

Yes/No

6b To your knowledge, would the outcome of the meeting or work benefit or adversely affect interests of others with whom you have substantial common personal, professional, financial or business interests (such as your adult children or siblings, close professional colleagues, administrative unit or department)?  

Yes/No

6c Excluding WHO, has any person or entity paid or contributed towards your travel costs in connection with this WHO meeting or work?  

Yes/No

6d Have you received any payments (other than for travel costs) or honoraria for speaking publicly on the subject of this WHO meeting or work?  

Yes/No

6e Is there any other aspect of your background or present circumstances not addressed above that might be perceived as affecting your objectivity or independence?  

Yes/No

7. TOBACCO OR TOBACCO PRODUCTS (answer without regard to relevance to the subject of the meeting or work)

Within the past 4 years, have you had employment or received research support or other funding from, or had any other professional relationship with, an entity directly involved in the production, manufacture, distribution or sale of tobacco or tobacco products or representing the interests of any such entity?  

Yes/No

EXPLANATION OF “YES” RESPONSES

If the answer to any of the above questions is “yes”, check above and briefly describe the circumstances on this page. If you do not describe the nature of an interest or if you do not provide the amount or value involved where relevant, the conflict will be assumed to be significant.
<table>
<thead>
<tr>
<th>Nos. 1–4; 7</th>
<th>Name of company, organization, or institution</th>
<th>Belongs to you, a family member, employer, research unit or other?</th>
<th>Amount of income or value of interest (if not disclosed, is assumed to be significant)</th>
<th>Current interest (or year ceased)</th>
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<tr>
<td>Type of interest, question number and category (e.g. Intellectual Property 4.a copyrights) and basic descriptive details</td>
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**CONSENT TO DISCLOSURE**

By completing and signing this form, you consent to the disclosure of any relevant conflicts to other meeting participants and in the resulting report or work product.

**DECLARATION**

I hereby declare on my honour that the disclosed information is true and complete to the best of my knowledge.

Should there be any change to the above information, I will promptly notify the responsible staff of WHO and complete a new declaration of interest form that describes the changes. This includes any change that occurs before or during the meeting or work itself and through the period up to the publication of the final results or completion of the activity concerned.

Date: ________________ Signature _________________________________
## Appendix 5

### Testing approach for initial evaluation for prequalification

<table>
<thead>
<tr>
<th>Category</th>
<th>WHO requirements/testing approach</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Novel vaccine or new combination released by a competent NRA/NCL</td>
<td>Requirements before prequalification is granted</td>
<td>• Detailed standard operating procedures for manufacturing and testing the product characteristics (relevant tests) for the regulatory oversight; NCL is performing the critical tests on a regular basis</td>
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<td>Requirements post-prequalification is granted</td>
<td>• Commitment from the manufacturer to keep retention samples for testing by WHO-contracted laboratories</td>
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<td>• Testing of the vaccine through the targeted testing programme</td>
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<td>• Review of United Nations tender aspects through samples of the vaccine in the final packaging presentation (to be submitted with the PSF)</td>
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<td>• Review of the testing results by the manufacturer and the NCL (raw data) of at least three lots formulated from consecutive bulk lots</td>
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<td>• Review of the trends of the testing results of both NCL and manufacturer (if applicable)</td>
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<td>• Review of the control chart of the reference used in manufacturers' and NCL's assays</td>
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<td>• Review of the method validation of the manufacturer and the NCL may be required</td>
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<td></td>
<td>• Review of the method validation of the manufacturer and the NCL may be required</td>
</tr>
</tbody>
</table>
### Category Criteria

**Novel vaccine released by a competent NRA/NCL responsible for the regulatory oversight; Validation of the critical tests is in progress**

<table>
<thead>
<tr>
<th>WHO requirements/testing approach</th>
<th>Requirements from the manufacturer before prequalification is granted</th>
<th>Requirements post-prequalification</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Review of United Nations tender aspects through samples of the vaccine in the final packaging presentation (to be submitted with the PSF)</td>
<td>• Detailed standard operating procedures for testing the product characteristics (relevant tests)</td>
<td>• Commitment from the manufacturer to keep retention samples for testing by WHO-contracted laboratories</td>
</tr>
<tr>
<td>• Review of the testing results by the manufacturer (raw data) of at least three lots formulated from consecutive bulk lots</td>
<td>• Biological reagents and reference materials for the validation of the tests by WHO-contracted laboratories</td>
<td>• Testing of the vaccine through the targeted testing programme</td>
</tr>
<tr>
<td>• Review of the trends of the testing results of the manufacturer (if applicable)</td>
<td>• Transfer of the relevant method by the manufacturer to the relevant laboratories through WHO</td>
<td></td>
</tr>
<tr>
<td>• Agreement with the NCL to validate the tests during the prequalification evaluation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Review of the method validation of the manufacturer and the control chart of the reference used in the manufacturer’s assays may be required</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Agreement to perform and provide results to WHO before prequalification is granted</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table continued

<table>
<thead>
<tr>
<th>Category</th>
<th>Criteria</th>
<th>WHO requirements/testing approach</th>
<th>Requirements from the manufacturer before prequalification is granted</th>
<th>Requirements post-prequalification</th>
</tr>
</thead>
</table>
| **III**  | Traditional vaccine released by a competent NRA/NCL responsible for the regulatory oversight; NCL is performing the critical tests on a regular basis | • Review of United Nations tender aspects through samples of the vaccine in the final packaging presentation (to be submitted with the PSF)  
• Review of the testing results by the manufacturer and the NCL (raw data) of at least three lots formulated from consecutive bulk lots  
• Review of the trends of the testing results of both the NCL and the manufacturer  
• Review the control chart of the reference used in the manufacturer’s and the NCL’s assays | • Detailed standard operating procedures for testing the product characteristics (relevant tests)  
• Biological reagents and reference materials for the tests by WHO-contracted laboratories  
• Testing of the vaccine through the targeted testing programme | • Commitment from the manufacturer to keep retention samples for testing by WHO-contracted laboratories |
| **IV**   | Novel or traditional vaccine NRA/NCL responsible for the regulatory oversight does not perform the critical tests | • Review of United Nations tender aspects through samples of the vaccine in the final packaging presentation (to be submitted with the PSF)  
• Testing by WHO-contracted laboratories before the prequalification is granted  
• Agreement with the NCL to validate the tests | • Detailed standard operating procedures for testing the product characteristics (relevant tests) |
<table>
<thead>
<tr>
<th>Category</th>
<th>WHO requirements/testing approach</th>
<th>Requirements from the manufacturer before prequalification is granted</th>
<th>Requirements post-prequalification</th>
</tr>
</thead>
</table>
| IV       | • Review of the testing results by the manufacturer (raw data) of at least three lots formulated from consecutive bulks lots  
          • Review of the control chart of the reference used in the manufacturer’s assays  
          • For novel vaccines, review of the method of validation of the manufacturer | • Biological reagents and reference materials for the validation of the tests by WHO-contracted laboratories  
          • Transfer of the relevant method (if applicable) by the manufacturer to the relevant laboratories through WHO |
Appendix 6

Prequalification procedure for vaccines evaluated by the EMA under Article 58 of Regulation (EC) No. 726/2004

Background

WHO provides a service to UNICEF and other United Nations agencies that purchase vaccines, to determine the acceptability, in principle, of vaccines from different sources for supply to these agencies.

The purpose of the prequalification assessment is to verify that the vaccines meet the specifications of the relevant United Nations agency, and are produced and overseen in accordance with the principles and specifications recommended by WHO for GMP, and for GCP. This is to ensure that vaccines used in national immunization services in different countries are safe and effective for the target population at the recommended schedules, and that they meet particular operational specifications for packaging and presentation.

For vaccines (and all medicines) manufactured by European manufacturers (or at least those with a legal presence in the European Community) intended for exclusive use in markets outside the European Community, the EMA established a mechanism (Article 58 of Regulation (EC) No. 726/2004) whereby the EMA may give a scientific opinion, in the context of cooperation with WHO.

WHO recognizes that the evaluation by EMA under Article 58 is conducted according to the principles applied by the prequalification process in terms of assurance of quality, safety and efficacy for the intended population (i.e. developing). WHO provides input at different stages of the process, including determination of eligibility of the product for evaluation under Article 58 and involvement in the assessment of the dossier. Therefore, in order to align the EMA evaluation under Article 58 and the WHO evaluation for prequalification purposes, a simplified procedure has been developed.

Application process to WHO

The applicant must submit the following:

1. An application letter is to be sent to the Coordinator, Quality, Safety and Standards, Department of Essential Medicines and Health Products at WHO, with a copy to the vaccines prequalification manager and the EMA, with details of the country and sites of manufacture and presentations offered.
Application letters can be sent at any time after the submission of the dossier to the EMA. Manufacturers are encouraged to advise WHO as early as possible of their intention to submit a specific vaccine application to facilitate planning.

(2) A statement that the applicant acknowledges and agrees to the fact that the EMA will share the report of the CHMP evaluators, inspection reports (manufacturing facilities and clinical trial sites) and test results, if available, with the WHO prequalification team, as well as mutual immediate notification of quality or safety concerns of the product.

(3) An electronic copy of the dossier submitted to the EMA for evaluation under Article 58.

(4) Technical information relevant to United Nations specifications, including information relevant to the programmatic suitability of the vaccine.

(5) Notification about the official medical control laboratory (OMCL) selected for any testing required by the EMA for evaluation under Article 58 or for prequalification by WHO.

(6) Fees (see section 14).

The evaluation process

WHO will base the evaluation on the following:

- the EMA Article 58 scientific opinion and its annexed assessment report from EMA/CHMP;
- a certificate of analysis of consistency lots by a qualified (OMCL) laboratory;
- reports from relevant inspections (GMP, GCP and good laboratory practice) jointly agreed by WHO and the EMA and performed during the EMA/CHMP evaluation procedure for Article 58 scientific opinion.

Although the EMA/CHMP procedure under Article 58 of Regulation (EC) No. 726/2004 is done by rapporteur/co-rapporteur, in collaboration with WHO and its experts/expert groups, with the evaluation ensuring that the clinical data provided by the applicant is relevant to the United Nations target population at the intended schedules, other programmatic aspects reflected in the tender specifications of the United Nations purchasing agencies will not be part of the review process under Article 58 evaluation and will therefore remain to be reviewed by WHO during the streamlined prequalification evaluation.
In view of the above, a number of reviews by WHO will remain essential, namely:

- confirmation that the vaccine meets the WHO recommendations and United Nations tender specifications;
- review of stability data to ensure they meet the needs of immunization programmes in developing countries (particularly those with weak cold-chain systems) and to assign a VVM category;*
- review of recommended immunization schedules to ensure compatibility with those existing in national immunization programmes and non-interference with co-administered vaccines;*
- review of samples, labels, inserts and packaging to suit the United Nations agency tender requirements;*
- review of mandatory, critical and innovative product characteristics from the programmatic point of view;*
- review of packaging for international shipment and its validation;
- if applicable, recommendation that the vaccine would be eligible for the AMC through review of the proposed product characteristics against the target product profile criteria.

**Note:** The items marked * are expected to be included in the EMA/CHMP evaluation done in collaboration with WHO under Article 58 of Regulation (EC) No. 726/2004. If such assessment and supportive data are available, the applicant should state so and should indicate specifically where these have been addressed in EMA/CHMP Article 58 scientific opinion documents.

**Report and outcome of the assessment**

Once WHO considers that the process is complete, and if the outcome is satisfactory, WHO sends a letter to UNICEF and other United Nations agencies, advising on the compliance of the vaccine with both the WHO recommendations and the specifications of the relevant United Nations agency. The vaccine will then be included in the WHO list of prequalified vaccines immediately after the letter to United Nations agencies is sent. The current list may be consulted at: http://www.who.int/immunization_standards/vaccine_quality/PQ_vaccine_list_en/en/index.html.

The prequalified status of a vaccine is valid until revoked by WHO.

**Assurance of continued acceptability**

After the prequalification of the product has been granted, follow-up activities to ensure continued acceptability of the vaccine for supply through United Nations
agencies will be performed according to the general prequalification procedure, as follows:

- reassessments;
- evaluation of variations submitted by the applicant;
- targeted testing of lots supplied to United Nations agencies;
- monitoring of continued compliance with specifications;
- follow-up of complaints and reports of AEFI.

The above list is indicative but not exhaustive. Failure of manufacturers to submit variations through the EMA may lead to withdrawal of the scientific opinion and the prequalification status.

These activities will be conducted, whenever applicable, in collaboration with the EMA within the context of Article 58 of Regulation (EC) No. 726/2004.
Annex 7

Biological substances: International Standards and Reference Reagents

A list of International Standards and Reference Reagents for biological substances was issued in WHO Technical Report Series, No. 897, 2000 (Annex 4) and an updated version is available on the Internet at http://www.who.int/biologicals. Copies of the list may be obtained from appointed sales agents for WHO publications or from WHO Press, World Health Organization, 20 avenue Appia, 1211 Geneva 27, Switzerland (tel.: +41 22 791 3264; fax: +41 22 791 4857; e-mail: bookorders@who.int; web site: http://www.who.int/bookorders).

At its meeting in October 2010, the Expert Committee made the following changes to the previous list.

The Transfusion-relevant bacterial strain repository is held and distributed by the Paul-Ehrlich-Institute, 63225 Langen, Germany. The Second International Standard for vancomycin is held and distributed by the European Directorate for the Quality of Medicines & Healthcare (EDQM), Council of Europe, 7 Allée Kastner, CS 30026, F-67081 Strasbourg, France. The other substances are held and distributed by the International Laboratory for Biological Standards, National Institute for Biological Standards and Control, Potters Bar, Herts, EN6 3QG, England.

Additions

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Activity</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antibiotics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vancomycin</td>
<td>109 700 IU per vial</td>
<td>Second International Standard</td>
</tr>
<tr>
<td><strong>Blood products and related substances</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preparations of four bacterial strains</td>
<td>No assigned values</td>
<td>Transfusion-relevant bacterial strain</td>
</tr>
<tr>
<td>(Staphylococcus epidermidis, Klebsiella</td>
<td></td>
<td>repository</td>
</tr>
<tr>
<td>pneumoniae, Streptococcus pyogenes,</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Escherichia coli)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antithrombin, plasma</td>
<td>Antithrombin function 0.95 IU/ampoule; antithrombin antigen 0.96 IU/ampoule</td>
<td>Third International Standard</td>
</tr>
<tr>
<td>Preparation</td>
<td>Activity</td>
<td>Status</td>
</tr>
<tr>
<td>-------------------------------------------------</td>
<td>--------------------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>C1 inhibitor, plasma</td>
<td>0.89 IU per ampoule</td>
<td>First International</td>
</tr>
<tr>
<td>C1 inhibitor, concentrate</td>
<td>9.6 IU per ampoule</td>
<td>First International</td>
</tr>
<tr>
<td>Factors II, VII, IX, X, plasma</td>
<td>Factor II: 0.89 IU/ampoule; factor VII: 0.99 IU/ampoule; factor IX: 0.86 IU/ampoule; factor XI: 0.89 IU/ampoule</td>
<td>Fourth International Standard</td>
</tr>
<tr>
<td>von Willebrand factor, concentrate</td>
<td>VWF:Ag 10.7 IU/ampoule; VWF:RCo 9.2 IU/ampoule; VWF:CB 10.3 IU/ampoule</td>
<td>Second International Standard</td>
</tr>
<tr>
<td><strong>Cytokines, growth factors and endocrinological substances, and related substances</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Follicle-stimulating hormone, human, recombinant, for bioassay</td>
<td>126 IU/ampoule</td>
<td>Second International Standard</td>
</tr>
<tr>
<td>Granulocyte colony-stimulating factor</td>
<td>95 000 IU/ampoule</td>
<td>Second International Standard</td>
</tr>
<tr>
<td>Sex hormone-binding globulin</td>
<td>180 IU/ampoule</td>
<td>Second International Standard</td>
</tr>
<tr>
<td>Thyroid-stimulating antibody</td>
<td>113 mIU/ampoule</td>
<td>Second International Standard</td>
</tr>
<tr>
<td><strong>Diagnostic reagents</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human cytomegalovirus for nucleic acid amplification technique-based assays</td>
<td>5 million IU/ml</td>
<td>First International Standard</td>
</tr>
<tr>
<td>RHD/SRY plasma DNA</td>
<td>No assigned value</td>
<td>First Reference Reagent</td>
</tr>
<tr>
<td><strong>Vaccines and related substances</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antibody to influenza A/California/7/2009 (H1N1) virus</td>
<td>1300 IU/ampoule</td>
<td>First International Standard</td>
</tr>
<tr>
<td>BCG vaccine, Russian BCG-I substrain</td>
<td>3.39 million cfu per ampoule; 7.5 ng ATP per ampoule</td>
<td>First Reference Reagent</td>
</tr>
<tr>
<td>Tetanus vaccine</td>
<td>490 IU/ampoule</td>
<td>Fourth International Standard</td>
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</table>