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 production and control of live attenuated influenza vaccines and for production and control of pneumococcal conjugate vaccines. New WHO Guidelines on the regulatory evaluation of similar biotherapeutic medicines are also provided.

Also included are a list of Recommendations, Guidelines and other documents for biological substances used in medicine, and of International Standards and Reference Reagent for biological substances.
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Fifty-ninth report.
WHO Technical Report Series, No. 964, 2012 (viii + 228 pages)
Web site: www.who.int/biologicals

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)

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Fifty-sixth report.
WHO Technical Report Series, No. 941, 2007 (x + 340 pages)

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WHO Expert Committee on Biological Standardization

Sixtieth 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.
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WHO Expert Committee on Biological Standardization
19 to 23 October 2009

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Introduction

The WHO Expert Committee on Biological Standardization met in Geneva from 19 to 23 October 2009. The meeting was opened on behalf of the Director-General by Ms Daisy Mafubelu, Assistant Director-General of the Family and Community Health cluster. She invited the members and the observers to introduce themselves.

Ms Mafubelu began by emphasizing the importance of the activities of the Committee and the impact of its work globally. The Committee assists the Organization in fulfilling one of its constitutional responsibilities, namely to “develop, establish and promote international standards for biological products”. The Committee advises the Organization on international biological standardization and key developments affecting the quality, safety and efficacy of vaccines, biological therapeutics, blood products and biological diagnostics. It is apparent that more innovation in biological medicines is occurring in more countries than ever before. Despite technological advances, controlling the quality, safety and efficacy of biologicals remains difficult and highly specialized. Furthermore, the supply chain for biological medicines is increasingly complex and international in nature. Finally, many countries have regulatory systems with insufficient capacity to provide appropriate oversight of new biologicals of potential benefit to public health, or oversight of biological components in the supply chain. Strengthening biological standardization and its implementation, in particular in emerging economies, remains a fundamental function of WHO, which translates into benefits for global public health.

Dr Philip Minor was elected as Chairman of the plenary sessions of the meeting, Dr Harvey Klein as Vice-chairman and Mrs Teena Jivapaisarnpong as Rapporteur. The meeting would divide into parallel tracks. Dr Minor was elected as Chairman, Mrs Jivapaisarnpong as Vice-chairman and Dr Derek Calam as Rapporteur for the vaccine and biotherapeutics track, and Dr Klein as Chairman, Dr Paul Strengers as Vice-chairman and Professor G. Vyas as Rapporteur for the blood products and related substances track. The Committee adopted the agenda (WHO/BS/09.2127) and the timetable proposed.
General

Strategic directions in biological standardization

The Expert Committee was informed of WHO priorities with respect to international biological standardization in 2009 and current global public health needs, together with considerations for future needs in international standardization in preparation for 2020.

A “biological” analyte is considered by WHO as one “of biological origin, which cannot be characterized adequately by chemical and/or physical means alone” (WHO Technical Report Series, No. 800, 1990, pp. 181–213). This is a practical definition, relating to the structural complexity of the material being standardized and to the current utility of analytical methods. One consequence of this definition is that a unit of biological activity can only effectively be described relative to a reference material, and not in terms of an absolute response (such as Système international (SI) units). In 2009, there were more than 250 WHO biological reference standards available, which are used to define the International Unit of biological activity for these analytes. In addition to global measurement standards, WHO also establishes global written standards, and promotes global consensus in terms of standardization of assays, development and refinement of quality control (QC) tests, and the scientific basis for setting specifications.

Two examples highlighted the role of international biological standardization in global public health crises. In the case of the 2009 pandemic of H1N1 influenza, international standards, such as agreed technical specifications for vaccine potency and reagents to measure vaccine potency, were critical to enable the international supply of H1N1 vaccines. Moreover, the international standard-setting process followed in developing these standards also created a community of experts who worked together in real time very effectively to address quality, safety and standards issues associated with H1N1 pandemic influenza vaccines.

The second example concerns a heparin contamination event in 2007–2008 which had been notified to Member States through the International Health Regulations (2005). This event had led to increased requests for the Fifth International Standard for unfractionated heparin, leading to near depletion of stock, and emphasizing the need for such materials.

Looking ahead to 2020, the Committee discussed whether the concept of a “biological” (not characterizable by physicochemical means alone) would still be valid and whether the 2009 construct of international biological standardization would still be valid. The Committee supported a proposal for an independent review of international biological standardization, that would also strengthen the impact of the ECBS, to take these matters forward.
Vaccines and biological therapeutics: recent and planned activities in biological standardization

The Committee was apprised of progress made since its previous meeting. Four written standards had been drafted and were to be presented to the Committee during the meeting. A further 11 written standards were under revision. A consultation had been held in April 2009 to discuss key issues to be taken into account in the revision of the current WHO Recommendations on animal cells, use of, as in vitro substrates for the production of biologicals (WHO Technical Report Series, No. 878). A draft document reflecting these discussions had been prepared and circulated for comment. A similar situation applied to revision of the Recommendations for yellow fever vaccine (WHO Technical Report Series, No. 872), for dengue vaccine (WHO Technical Report Series, No. 932) and for BCG vaccines (WHO Technical Report Series, No. 745, and amendment WHO Technical Report Series, No. 771). A schedule for submission of the revision of the proposed Recommendations to the Committee was agreed.

The Committee was informed that a meeting of WHO collaborating centres involved in biological standardization had been held, and regular meetings were planned for the future. Proposals for international reference materials for submission to the Committee had been reviewed and various issues resolved. Proposals for future projects on reference materials were also considered, bearing in mind the need for a balance between scientific issues and public health needs. A collaborative approach to achieve joint project and work planning had been discussed. Designation of new centres was being pursued to diversify the support available to WHO through its collaborating centres. The Committee recommended that WHO develop further synergies between WHO collaborating centres in the vaccines and biotherapeutics areas.

A meeting, involving regulators and industry, had been held to discuss implementation of the WHO Guidelines for thermal stability evaluation of vaccines (WHO Technical Report Series, No. 962) and the new paradigm for such evaluations described in the Guidelines. The meeting report had been published. The Committee recommended that such post-adoption workshops should be continued as a way of promoting the implementation of written standards and that consideration should be given to such an approach for reference materials.

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

The Committee was informed of recent progress made with WHO Biological Reference Preparations and with Guidelines and Recommendations. Discussions took place on some questions of access and implementation and on advice on the way forward.
The Committee was reminded that WHO Biological Reference Preparations are global reference standards and, as such, are tools for comparison of biological measurement results worldwide. The reference preparations facilitate transfer of laboratory science into worldwide clinical practice and underpin appropriate clinical dosage of biological therapeutics. As global standards they support harmonization of international regulations and accelerate transfer of knowledge between countries. The Committee has prioritized establishment of global reference standards for human blood-derived products and other related products, such as anticoagulant and fibrinolysis biological therapeutic products, and for selected animal-derived sera used in human medicine. The Committee was reminded that blood-derived medicinal products for the treatment of haemophilia and immune diseases are included in the WHO Model List of Essential Medicines.

In the area of in vitro biological diagnostic devices (IVDs), the priority has been to establish reference preparations that support international regulations. Specifically for IVDs for infectious markers, the aim of the WHO biological standardization programme is to prioritize reference preparations that define analytical test sensitivity in order to provide a tool for inter-laboratory data linkage and to help in setting up international quality control programmes. The intended use of such reference preparations is in the fields of blood products safety and in vitro clinical diagnostics.

WHO coordinates these standard-setting activities through the workplans of the WHO collaborating centres that develop IVD biological reference preparations. WHO infectious disease control programmes provide overviews of global epidemiological data. WHO regional offices help to identify participating laboratories for collaborative studies and in the identification of candidate materials for evaluation as reference preparations. WHO also collaborates with other standard-setting organizations and international organizations to minimize duplication of work.

The Committee agreed with a proposal to develop a new WHO Guidelines on good manufacturing practices for blood establishments, and looked forward to receiving the proposed Guidelines at its next meeting. The Committee was also informed that the WHO Guidelines on tissue infectivity distribution in transmissible spongiform encephalopathies were intended to assist regulatory authorities and manufacturers in conducting risk assessment studies and selecting measures to reduce the risk of transmitting transmissible spongiform encephalopathies (TSEs) through medicinal products. A proposed update based on new scientific information had been compiled by an expert advisory group and would be considered by the Committee.

The Committee was also presented with the outcomes of a survey on regulation of antivenoms in Africa and Asia. Some basic problems in the production of venoms were highlighted. For example, venoms used for production...
need to be representative of the snake populations in the territories where the antivenoms will be distributed. Further, technical capacity needs to be generated to assess the neutralizing potency of antivenoms for preclinical assessments of products. The Committee agreed that reference preparations for venoms may be a key component in any global strategy aimed at increasing the production and use of effective and safe antivenoms.

The Committee supported:

- further work to coordinate and define priorities for global public health for WHO standard-setting activities for blood products and related biologicals;
- development of a common policy for distribution of reference materials by different custodian laboratories; and
- the proposed workplan for new and revised written standards.

**Blood Regulators Network**

The chairman of the Blood Regulators Network provided a brief history of the Network and presented an overview of developments during the past year. He reminded the Committee that the Network had been established between six control and regulatory authorities in 2006 to serve as an expert group to coordinate comments and proposals being submitted to WHO. Teleconferences were held quarterly and an extra virtual meeting was held during the emergence of pandemic H1N1 2009 influenza virus. Comments were sent on draft WHO documents and a position paper was submitted on collection and use of convalescent plasma as part of the planned response to pandemic influenza. Work was in progress on the assessment criteria for national blood regulatory systems. Information was being shared on topics which included a proposed World Health Assembly (WHA) resolution on quality, safety and availability of blood products, standardization of some assays, and implications of emerging arbovirus infections. The interest of other countries in joining the Network has indicated the need to establish suitable criteria for membership. The Committee recommended that criteria be developed to expand membership of the network and supported development of a tool for standardized assessment of regulatory capacity for blood products.

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

The Committee was informed of recent developments at the various WHO international laboratories and collaborating centres for biological standards.
National Institute for Biological Standards and Control, Potters Bar, England

The Committee was provided with an overview of current activities and developments at the National Institute for Biological Standards and Control (NIBSC) concerning the WHO programme for biological standardization. A merger with the Health Protection Agency (HPA) had resulted in the transfer of responsibility for NIBSC to the HPA Board. The Board has recognized the importance of the international work of NIBSC and it was hoped that the merger would not affect this activity. The Committee was provided with details of a new building at NIBSC to accommodate work for the stem cell bank and related research in the United Kingdom, and also to house activities concerning influenza vaccines, including provision of biocontainment facilities, and for production of candidate vaccine strains and associated reagents for calibration of vaccine potency.

Activities surrounding aspects of pandemic H1N1 influenza had been a major task of NIBSC in 2009. Production of vaccine strains of virus had played a critical role in the development of vaccines suitable for manufacture. The first strains had low yields but new higher-yielding strains had been successfully developed. Work on the development of an international standard for assay of the antibody response to H1N1 vaccines was outlined. The Committee was also informed of the problem of contamination of heparin in some countries, and the important international role which NIBSC had played during scientific assessment of the event. Areas of growth for the Institute included control and standardization of cell-based medicines. Quality assurance of similar biotherapeutic products was an area in which application of reference materials is likely to play an important part. The role of clinical diagnosis employing nucleic acid amplification testing (NAT) and genetic screening is increasing and will have significant implications for sourcing and preparing appropriate international reference materials. A new group had been formed within the Institute to undertake work in this area of strategic importance.

During the past year, approximately 23 000 vials and ampoules of WHO reference materials had been shipped. The development and demand for reference materials was giving rise to increasing problems with the storage of existing and new stocks. In 2008, the excess of storage over use at NIBSC was more than 350 000 units. Some off-site storage is used, partly for reasons of security as well as space, but this incurs significant costs. There are excessive stocks of little-used reference materials. It was suggested that stocks to cover estimated usage for 30 years should be adopted as a guide to holdings required. It may be important to consider developing guidance for custodian laboratories on selective reduction of stocks of such materials. The Committee was informed that 16 new and replacement reference materials from NIBSC, covering vaccines,
biotherapeutic and blood products and genetic panels, were on the agenda for the current meeting. In addition, 21 new projects, especially in the diagnostics area, were being submitted for approval by the Committee.

The Committee commended the work of NIBSC in the field of international biological standardization and requested the secretariat to work with NIBSC to develop solutions to the issue of storage space for WHO reference materials.

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

The Committee was informed of changes and activities at the Paul-Ehrlich-Institute (PEI). The name of the Institute had changed to the Federal Institute for Vaccines and Biomedicines. The responsibilities of the Institute include in vitro diagnostics, cell and gene therapy products, xenotransplantation, tissue repair, and stem cells. PEI had contributed to WHO projects on quality assurance and safety of blood products and on in vitro diagnostics as well as to the WHO Blood Products Network. It had played a leading role in the proposal to put a resolution on assurance of safety and availability of blood-derived medicinal products before the WHO Executive Board.

The Committee was also informed about the contributions PEI had made to its activities, including organization of, and participation in, collaborative studies and development of reference materials, especially for antibodies to hepatitis B core antigen, in the field of in vitro diagnostics, and the hepatitis B virus and parvovirus B19 genotype panels. PEI was also evaluating bacterial strains for the development of blood bacteria reference panels for screening blood components to reduce adverse effects. It hoped to present a proposal to the Committee in 2010. PEI also provides training courses for assessors from regulatory authorities globally. The Committee requested PEI to provide feedback to WHO on the impact of the training course for assessors working in regulatory authorities.

**Center for Biologics Evaluation and Research (CBER), Food and Drug Administration, Bethesda, USA**

The Committee was informed of the mission and functions of the Division of Product Quality (DPQ) at CBER. These include a range of activities relating to laboratory testing of products during the process of approval as well as lot testing of approved products. Testing capability was being further developed and it was planned to pursue laboratory accreditation. An independent Quality Assurance Group within DPQ oversees all quality functions and would play a key part in achieving these aims. DPQ interacts with the processes for licensing and lot release in other parts of CBER. Areas of interest include developing alternative rapid methods to enable faster availability of products during emergencies, developing
rapid methods for detection of mycoplasma, advanced immunochemical methods, and investigating genetic stability of viral vaccines.

CBER’s activities in the field of emerging infectious diseases were also outlined. There is concern about the threat to blood safety from viruses which cause asymptomatic viremia. Other emerging infections of concern include West Nile virus and dengue. Assessment of risks was to be addressed in a workshop of the United States Food and Drug Administration (FDA) in December 2009. Steps were being taken to prepare and establish an RNA standard for NAT of dengue viruses, using isolates of the four serotypes. Work was also in hand to develop an antibody reference panel for babesia infections. Finally, the Committee was informed of work aimed at developing reference materials useful in studying variant Creuzfeldt–Jakob disease (vCJD).

**Reports from international groups establishing secondary standards**

Links between the European Directorate for Quality of Medicines and Healthcare (EDQM) and WHO were described. As well as producing the *European Pharmacopoeia*, EDQM has specific responsibility in the biological field for European reference materials, calibrated against WHO primary reference materials, and for a programme on biological standardization concerned with assay method development, overseen by a Steering Group on which WHO sits. The Committee was informed that 111 projects had been initiated, 33 on method development and 78 on reference materials. Two projects intended to replace in vivo with in vitro assays, for diphtheria vaccine and for factor VIII, had been completed successfully. EDQM also runs the Official Medicines Control Laboratories Network responsible for batch (lot) release of vaccines. EDQM is custodian of the WHO reference materials for antibiotics, on which no developments were reported.

Some of the biological standardization projects involve joint collaboration between WHO and EDQM. Therefore, projects initiated by EDQM that were of possible interest to the Committee were outlined. Three involved pertussis. The first was a test for pertussis toxin. The second was a serological assay for acellular pertussis vaccine, which had shown that all the components can be assayed in a single animal resulting in a significant reduction in the use of animals, and in revised pharmacopoeial requirements. The third project was the replacement of the Kendrick test by a serological assay for whole cell vaccine which was at a preparatory stage. A project was described that aimed to replace a challenge test by a serological assay for rabies vaccine for veterinary use, and may have implications for the human rabies vaccine. A project aiming to replace a challenge test by an in vitro assay for tetanus immunoglobulin was also described.
Issues shared with the WHO Expert Committee on Specifications for Pharmaceutical Preparations

The Committee was informed of the outcomes of the recent meeting of the WHO Expert Committee on Specifications for Pharmaceutical Preparations at which a number of matters of common interest were discussed. New and revised texts had been adopted, including Recommendations on sterile products and on hazardous products. The Expert Committee had also been given an update on global priorities of primary health care and on International Nonproprietary Names. High priority was being given to finding effective anti-counterfeiting measures and a survey to provide an information base was about to be initiated.

Pharmaceutical cold-chain management

At its previous meeting, the Committee had noted that effective distribution of vaccines that are sensitive to temperature is a key factor in ensuring that vaccination programmes achieve their objectives. The Committee was reminded of the aim of WHO to provide guidance on the storage and transport of temperature-sensitive pharmaceutical products. WHO had assembled and analysed the contents of a large number of documents that had been published on this topic and, in some cases, implemented. A task force had been established, drawn from countries in many regions of WHO and supported by a Secretariat, to review existing documents, identify overlapping and conflicting areas and aspects that were missing. Four drafts had been produced before a mature draft version was provided to many stakeholders for comment. It was intended to form an advisory group in 2010 to finalize the draft which would then be submitted to the Expert Committee on Biological Standardization and the Expert Committee on Specifications for Pharmaceutical Preparations. The Committee welcomed this proposal and noted that it would be important to ensure input from blood products regulators and industry.

Nomenclature of stem cells

A request had been made to WHO to develop a global scheme for nomenclature of stem cells and other cell systems of human origin. There was concern about confusion of cell therapy products in the absence of a suitable nomenclature system. A small group had been formed to consider this issue in the light of existing nomenclature systems. Further investigation was planned and a further report would be provided to the Committees.
International Recommendations, Guidelines, and other matters related to the manufacture and quality control of biologicals

All Recommendations and Guidelines established at 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.

Guidelines for independent lot release of vaccines by regulatory authorities

The Committee was reminded of the importance of lot release of individual batches of vaccines, which have been licensed, performed by national regulatory authorities and control laboratories, in ensuring the quality and safety of vaccines. Guidelines for national regulatory authorities, including on lot release, had been available for some years (WHO Technical Report Series, No. 822) but revision was required to reflect current approaches. Discussions between regulators and industry had clarified areas of agreement on principles that the guidelines should reflect. Two drafts had been prepared by working groups. At its meeting in 2008, the Committee had considered the proposals and made some further suggestions. After revision, the draft was again circulated widely for comment, and was further modified taking these minor comments into account. The revisions addressed issues of cooperation and sharing of information between regulatory authorities and control laboratories, and reducing the number of animals used in testing.

The Committee reviewed the most recent draft proposals (WHO/BS/09.2109) in the light of the comments and made some further changes. The Committee concluded, however, that further work was required, and although it affirmed the urgent need for guidance, the Committee requested that a revised version be submitted at its meeting in 2010. The Committee requested the Secretariat to add a section on WHO lot-release policy in the context of prequalification of vaccines for supply to the United Nations, and to consult further, especially with non-producing countries.

Guidelines on evaluation of similar biotherapeutic products (SBPs)

The Committee was reminded that WHO had accepted a request from national regulatory authorities to develop guidance on considerations for regulatory approval of biotherapeutic products that are similar to existing licensed products. The Committee was also reminded that in 2008, it had requested that a revised version of the draft Guidelines for evaluation of similar biological products should be prepared, reflecting discussion by the Committee, and published for public comment. Further drafts had thus been prepared and discussed in two meetings of
interested parties and then circulated for public comment. A new version had been drafted and was presented to the Committee (WHO/BS/09.2110). Changes that had been made following the discussions in 2008 and arising from a second round of public comment were explained to the Committee. The role of the document was to provide a framework of guiding principles. Substantial changes had been made and the role of national regulatory authorities and a statistical section had been added. The new draft also reflected experience in this field in many countries. The Committee accepted the basic assumptions on which the draft had been based.

Having reviewed at length the content of the draft (WHO/BS/09.2110) and after having made some alterations to it, the Committee adopted the revised version as Guidelines on evaluation of similar biotherapeutic products and agreed that it should be annexed to this report (Annex 2).

Recommendations to assure the quality, safety and efficacy of pneumococcal conjugate vaccines

The Committee was informed of the need for a revision of the current WHO Recommendations for pneumococcal conjugate vaccines (WHO Technical Report Series, No. 927 (2005)). Since the adoption of the existing Recommendations, applications for marketing authorizations for these vaccines had raised a number of issues, for example, criteria for evaluation of candidate vaccines with a larger number of serotypes than the original product, and an increased range of protein carriers, which needed to be addressed. In addition, there had been advances in test methodology. A drafting group was formed and a meeting was held to identify the necessary changes to the Recommendations. A new draft had been prepared that had a modified structure and a number of detailed changes, particularly regarding serological criteria and post-marketing studies. The document was intended for regulatory authorities and manufacturers. The draft was circulated for comment and a revised draft prepared.

After reviewing the final draft (WHO/BS/09.2108) and making some alterations to it, the Committee adopted the revised text as Recommendations to assure the quality, safety and efficacy of pneumococcal conjugate vaccines and agreed that it should be annexed to this report (Annex 3).

Recommendations to assure the quality, safety and efficacy of live attenuated influenza vaccines

The Committee was informed of the need for a revision of the current WHO Recommendations for live (attenuated) influenza vaccines (WHO Technical Report Series, No. 638 (1979)). The Committee was reminded that the occurrence of pandemic influenza had resulted in the recognition that the 1979 Recommendations were in part no longer relevant or applicable. Since the first
live, attenuated vaccine had been developed, numerous other strains had been isolated, studied and used for vaccination. More had been learned about the detailed structures of the strains, about their genetic modification and their use in vaccine production. The revision of the Recommendations was intended to provide vaccine manufacturers and national regulatory authorities with guidance on specific processes for production and control as well as on nonclinical and clinical evaluation. It was not intended for a particular form of vaccine virus attenuation but for seasonal and pandemic vaccines as well as egg-grown and genetically modified strains. The draft had not specified the use of only specific-pathogen-free (SPF) eggs. It also envisaged the possible future use of cell culture substrates. It provides guidance on control of adventitious agents and on the monitoring of healthy flocks. It also provides guidance on technical support. A revised draft of the Recommendations had been circulated for comment and further modified in the light of comments received (WHO/BS/09.2111).

The Committee reviewed the draft and proposed some changes to it. The Committee adopted the revised text as Recommendations to assure the quality, safety and efficacy of influenza vaccines (human, live attenuated) for intranasal administration and agreed that it should be annexed to this report (Annex 4).

**Good manufacturing practices for manufacturing blood products for transfusion**

Draft WHO guidelines had been prepared by the Blood Products and Related Biologicals group of the WHO Essential Medicines and Pharmaceutical Policies Department, with the collaboration of experts and regulatory inspectors in the blood products field. The purpose at this stage was to invite comments and suggestions. The draft and the comments received were shared with the Committee. A total of 80 comments were received during the discussion. The comments, where appropriate, were incorporated into a new draft version. The Committee agreed with a proposal for public consultation and accepted an offer from the Blood Regulators Network to review the document after public consultation.

**Convalescent plasma**

A Blood Regulators Network position paper on collection and use of convalescent plasma or serum as an element in pandemic influenza planning was presented to the Committee. In discussion, questions were raised about the value of convalescent plasma or fractionated plasma from convalescent patients or vaccinated persons. It was also noted that the treatment, although therapeutically valuable, was not without risk to the patient regarding volume and dose. In some parts of the world, this type of experimental treatment would require clinical trials; however, it was noted that under exceptional pandemic circumstances, regulatory authorities may have discretionary powers and should be consulted.
Snake bite envenomings and antivenoms

Development of a WHO snake antivenoms web site had commenced in February 2009 and it was expected to be launched in January 2010. The web site would include the recently adopted WHO Guidelines for production, control and regulation of antivenom immunoglobulins. The web site would also have a searchable index linking to individual snake species, providing the taxonomic history, local names, geographical distribution maps, antivenom data, literature citations and photographs of the snakes. The web site would provide data sources for antivenoms, based on the information provided by the manufacturers from Africa and Asia. The progress and plans described were commended and endorsed by the Committee.
International reference materials – antigens and related substances

All reference materials established at the meeting are listed in Annex 5.

Inactivated poliovirus vaccines (trivalent)

The need had been identified for a replacement International Standard for the current Second International Standard for Inactivated Polio Vaccine, the stocks of which had become depleted and the stability of which had been questioned. In addition, there was scientific uncertainty as to whether a reference preparation based on wild-type poliovirus strains would be suitable to assay inactivated poliovirus vaccines manufactured from Sabin (attenuated) poliovirus strains. Candidate materials had been obtained and distributed into ampoules and a collaborative study had been organized.

The collaborative study (WHO/BS/09.2119) was performed by 14 laboratories from 11 countries, and included both national control laboratories and manufacturers. However, when the results were analysed, it became clear that participants had found significant differences in the determination of in vitro potency (D antigen content) determined by enzyme-linked immunosorbent assay (ELISA) for some samples. There were indications of reduced potency of the current International Standard. The results also showed that use of a reference preparation based on wild poliovirus strains to assay inactivated poliovirus vaccine (IPV) manufactured from Sabin strains was not satisfactory. It was recommended that another collaborative study should be organized which would include more samples and a range of in vitro and in vivo assays. Meanwhile it was recommended to cease distribution of the current International Standard and that another suitable preparation, the European Biological Reference Preparation batch 2, which had been calibrated against the International Standard, should be distributed in its place until a replacement WHO International Standard is established.

The Committee endorsed these proposals and also recommended that a scientific meeting be convened to agree on the way forward for a replacement International Standard, and on how to include a pathway for establishment of a Sabin-IPV reference preparation.

Poliovirus type 1 (Sabin)

The need had been identified for a new International Standard for use in the mutant analysis by the polymerase chain reaction and restriction enzyme cleavage (MAPREC) test for poliovirus type 1 to monitor consistency of vaccine production. Standards for use in the MAPREC test for poliovirus types 2 and 3
had been established previously. The need for reference reagents for inclusion in each assay was also recognized.

The candidate standard and one of the candidate reference reagents, for 100% 480A, 525C DNA, were prepared by CBER, FDA and filled at NIBSC in single runs into batches of approximately 1000 ampoules and freeze-dried. The coefficient of variation for the fills was 0.07% and 0.1% for the candidate standard and the candidate reference reagent respectively. The ampoules were stored at −20 °C. The candidate high-mutant and low-mutant virus reference reagents were also prepared by CBER, FDA. They were not freeze-dried but filled into vials as suspensions. Stability studies have established that there was no significant drop in estimates for percentage 480A, 525C in samples after storage for 2 years at up to +56 °C. The collaborative study (WHO/BS/09.2103) was performed by five laboratories in five countries and involved assay of the candidate materials and three virus test samples in the Type 1 MAPREC test. On the basis of the results of the collaborative study, the Committee adopted the preparation (code 00/418) as the First International Standard for MAPREC assay of poliovirus type 1 (Sabin) and assigned a value of 2.06% 480A, 525C DNA per ampoule to it.

The Committee also adopted the following preparations.

- The First International Reference Reagent for 100% 480A, 525C DNA, restriction enzyme cleavage control for MAPREC assay of poliovirus type 1 (Sabin) (coded 00/410) with an assigned value of 85.2% 480A, 525C DNA.
- The First International Reference Reagent for low-mutant virus reference (LMVR) for MAPREC assay of poliovirus type 1 (Sabin) (coded 00/416) with an assigned value of 1.84% 480A, 525C DNA.
- The First International Reference Reagent for high-mutant virus reference (HMVR) for MAPREC assay of poliovirus type 1 (Sabin) (coded 00/422) with an assigned value of 2.56% 480A, 525C DNA.

**Human papillomavirus type 16 antibodies**

The Committee was reminded that a preparation containing human papillomavirus type 16 antibodies had been submitted previously. The serum preparation, consisting of unique material of human origin, had been filled at NIBSC in one run into a batch of approximately 3000 ampoules and freeze-dried. The ampoules were stored at −20 °C and −70 °C. The residual moisture ranged from 2% to 15% in individual ampoules and the residual oxygen was 15–18%. Both ranges were outside usually acceptable limits. Nevertheless the preparation had been adopted as the First International Reference Reagent. Stability studies had established that there was no significant change in antibody titre on storage of different ampoules, with low and with high moisture content, for 14 months at +20 °C. On the basis of the results of the stability study, the Committee accepted
a proposal (WHO/BS/09.2113) to upgrade the status of the First International Reference Reagent to the First International Standard for antibodies to human papillomavirus type 16 (preparation code number 05/134) and assigned a content of 5 International Units per ampoule to it.

**Bordetella pertussis serotype 2 and serotype 3**

The need had been identified for International Standards for accurate and reliable serotyping of *Bordetella pertussis* antigens for use in validating commercial kits, in standardizing assays for monitoring the consistency of pertussis vaccine production strains and for serotyping clinical isolates.

Monoclonal antibodies to different serotype antigens can now be produced in tissue culture. Candidate preparations against fimbrial antigens 2 and 3 were obtained, filled and freeze-dried at NIBSC, in separate runs, into batches of approximately 7500 ampoules for serotype 2 and approximately 5250 ampoules for serotype 3.

The coefficient of variation for the fills was 0.23% for type 2 and 0.14% for type 3; the residual moisture was determined to be 0.29% for type 2 and 0.78% for type 3, and the residual oxygen was 0.15% for type 2 and 0.21% for type 3. The ampoules were stored at −20 °C. Stability studies have established that there was no significant change in the results of ELISAs after storage for 2 years at up to +37 °C.

The collaborative study (WHO/BS/09.2120) was performed by 12 laboratories in nine countries. It involved typing of strains with the candidate materials using different assay methods including ELISA. On the basis of the results of the collaborative study, the Committee adopted the preparation coded 06/124 as the First International Standard for serotyping *Bordetella pertussis* fimbrial antigen 2. The Committee also adopted the preparation coded 06/128 as the First International Standard for serotyping *Bordetella pertussis* fimbrial antigen 3. No potencies were assigned to these preparations.

**BCG vaccine**

The First International Reference Preparation of BCG vaccine was established in 1965. Stocks of the International Reference Preparation are now very low and the viability of this lyophilized, live BCG preparation has diminished. The Committee was informed that WHO had held three consultation meetings to discuss the replacement of the preparation. In order to provide appropriate material for vaccine manufacturers and national control laboratories to use in assays of vaccines, the recommendation was made to prepare separate reference reagents for different live bacterial sub-strains that are widely used in BCG vaccine manufacture.

Three different BCG vaccine manufacturers each donated 5000 ampoules of sterile, lyophilized, live BCG sub-strains. The data provided to NIBSC for each preparation were:
Danish sub-strain (coded 07/270): coefficient of variation for the fill, 0.45%; residual moisture, 3.1 %

Moscow sub-strain (coded 07/274): coefficient of variation for the fill, 2.18%; residual moisture content not provided

Tokyo 172 sub-strain (coded 07/272): coefficient of variation for the fill, 0.34 %; residual moisture 2.9%.

The ampoules were stored at −20 °C. The usual accelerated degradation studies are not applicable to these live preparations. Instead, the temperature stability assay after incubation at +37 °C for 4 weeks was performed and found satisfactory. Real-time monitoring of stability would continue. The collaborative study (WHO/BS/09.2114) was performed by 11 laboratories in 10 countries. Different assay methods were used:

- viable count for colony-forming units (CFU);
- multiplex polymerase-chain reaction (PCR) assay for identity; and
- adenosine triphosphate (ATP) assay for content.

All strains were correctly identified. It is intended that the materials will be used as comparators for determination of viable bacterial counts, and for control of the ATP viability assay. These strains are not to be used for production.

On the basis of the results of the collaborative study, the Committee adopted the preparations and assigned potencies and content as follows.

- First Reference Reagent for BCG Danish sub-strain (coded 07/270) with a CFU content of 7.29 × 10^6 and an ATP content of 56.06 ng per ampoule.
- First Reference Reagent for BCG Tokyo sub-strain (coded 07/272) with a CFU content of 49.37 × 10^6 and an ATP content of 217.60 ng per ampoule.

Adoption of the First Reference Reagent for BCG Moscow sub-strain (coded 07/274) with a CFU content of 3.39 × 10^6 and an ATP content of 7.52 ng per ampoule was subject to provision of satisfactory data on the moisture content.

**Diphtheria vaccine (adsorbed)**

The current Third International Standard for Diphtheria Toxoid (Adsorbed) was adopted in 1999 (WHO Technical Report Series, No. 904). Several combination vaccines containing diphtheria toxoid had been introduced since then, increasing demand for the Standard, stocks of which had become depleted. Two candidate materials were supplied to NIBSC. Each was stabilized by addition of an equal volume of Haemacel®, distributed into ampoules and lyophilized to give batches
of approximately 9900 ampoules. For the proposed standard, the coefficient of variation for the fill was 0.21%, the residual moisture was determined to be 0.40% and the residual oxygen was 0.04%. The ampoules were stored at −20 °C. Stability studies employing both in vitro and in vivo assay methods had established that there was no significant loss of activity after storage for 12 months at up to +56 °C. The collaborative study (WHO/BS/09.2123) was performed by 30 laboratories in 20 countries. This involved comparison of the two candidate materials against the existing material in in vivo challenge tests and serological assays. On the basis of the results of the collaborative study for challenge assays, the Committee adopted the preparation coded 07/216 as the Fourth International Standard for Diphtheria Toxoid (Adsorbed) and assigned a potency of 213 IU per ampoule to it.
International reference materials – blood products and related substances

All reference materials established at the meeting are listed in Annex 5.

International Society on Thrombosis and Haemostasis report

The Committee received a report from the International Society on Thrombosis and Haemostasis (ISTH) Standards Liaison Group. This is a formal subgroup of the ISTH Scientific and Standardization Committee (SSC). The Expert Committee was pleased to note the in-depth discussion of issues relevant to WHO, and the expert reviews of certain reference preparation proposals submitted to this meeting.

Blood coagulation factor VIII, concentrate

The current WHO Seventh International Standard for Factor VIII Concentrate (coded 99/678) and the European Pharmacopoeia (Ph. Eur.) Biological Reference Preparation Batch 3 (BRP 3) are used in potency estimation of Factor VIII (FVIII) in plasma-derived and recombinant therapeutic concentrates, which are primarily used in the treatment of haemophilia A (FVIII deficiency). Stocks of both standards were running low. NIBSC (a WHO collaborating centre) and the European Directorate for the Quality of Medicines and HealthCare (EDQM) had undertaken a joint project to replace these standards. Following trial-fill studies on a number of therapeutic FVIII concentrates, four candidate materials were selected based on fill characteristics, stability of FVIII potency, minimal inter- and intra-assay variability and minimal discrepancy between assay methodologies. Twenty thousand ampoules of each of the four candidate materials had been prepared. In the interests of harmonization and continuity, a joint international collaborative study was undertaken to calibrate the replacement of both the above standards.

The Committee reviewed the results from the joint international collaborative study, which involved 38 laboratories. Four candidate materials, two plasma-derived and two recombinant preparations, were evaluated. All laboratories were instructed to use their routine validated methods and to follow the recommendations of the SSC of the ISTH, including pre-dilution of concentrate samples in FVIII-deficient plasma. Laboratories returned data from one-stage clotting methods (22 datasets) and chromogenic methods (30 datasets).

On the basis of the study data one plasma-derived candidate was considered the favoured candidate for the following reasons:

- complete agreement in mean values obtained by the one-stage and chromogenic methods, whereas all other candidates displayed some discrepancies;
The collaborative study confirmed that the proposed International Standard would be suitable for measurement of all FVIII therapeutic concentrates. The excellent parallelism of assays for both methods, relative to all standards for FVIII concentrate included in the study, validates its suitability for both plasma-derived and full-length recombinant FVIII concentrates.

On the basis of the results of the collaborative study (WHO/BS/09.2117), the Committee adopted the preparation (NIBSC code 07/350) as the WHO Eighth International Standard for FVIII concentrate with a potency of 9.4 IU/ampoule.

Blood coagulation factor VIII and von Willebrand factor (VWF), plasma

The current WHO Fifth International Standard (coded 02/150) was established in 2003 and is used for the potency estimation of five analytes in plasma (factor VIII coagulant activity; factor VIII antigen; VWF antigen; VWF ristocetin cofactor and VWF collagen binding). The Fifth International Standard provides a common traceable source of calibration to promote harmonization between secondary working plasma standards and is primarily used for the estimation of these analytes in relation to the diagnosis of disease (e.g. haemophilia A and von Willebrand disease).

Stocks of the WHO Fifth International Standard were extremely low and a replacement preparation was required.

A collaborative study between 44 laboratories in 14 countries was conducted for the value assignment of a proposed WHO Sixth International Standard by assay relative to the WHO Fifth International Standard and locally collected, normal plasma pools. The results showed the candidate material to be fit for purpose. All of the 44 participants approved the proposed value assignments for the five analytes. Responses had also been sought from experts associated with the Factor VIII/Factor IX and von Willebrand factor sub-committees of the ISTH. All responses were in favour of the proposed value assignments.

Stability of the proposed Sixth International Standard had been assessed through an accelerated degradation study after storage of ampoules at elevated temperatures (+4 °C, +20 °C, +37 °C, and +45 °C) for up to 9 months. The results from this testing were very encouraging and indicated that all analytes in the proposed Sixth International Standard were extremely stable when stored at the bulk storage temperature of −20 °C with the mean predicted losses per
year of 0.074% for FVIII:C 1-stage, 0.146% for FVIII:C chromogenic, 0.049% for FVIII:antigen, 0.00% for VWF:antigen, 0.022% for VWF:ristocetin cofactor and 0.002% for VWF:collagen binding.

On the basis of the results of the collaborative study (WHO/BS/09.2116), the Committee adopted the preparation coded 07/316 as the WHO Sixth International Standard Factor VIII/von Willebrand Factor, Plasma and assigned potencies and content as follows:

- Factor VIII: C – 0.68 IU/ampoule
- Factor VIII: antigen – 1.04 IU/ampoule
- von Willebrand Factor: antigen – 1.00 IU/ampoule
- von Willebrand Factor: ristocetin cofactor – 0.87 IU/ampoule
- von Willebrand Factor: collagen binding – 1.03 IU/ampoule.

**Streptodornase**

The WHO First International Standard for Streptokinase and Streptodornase, 62/007, was established in 1964 by the Committee and stocks were almost exhausted. This International Standard was manufactured from a batch of streptokinase of medium purity that would serve as a reference standard for both streptokinase potency and streptodornase potency. Streptokinase is a thrombolytic plasminogen binding and activating protein used to treat myocardial infarction and streptodornase is a DNAse enzyme that may be a contaminant in streptokinase preparations. Manufacturers of streptokinase measure the streptodornase activity during production of streptokinase for therapeutic use and this activity must be below a cut-off level to fulfil pharmacopoeial requirements. For example, the European Pharmacopoeia specifies a maximum limit of 10 IU of streptodornase for 100 000 IU of streptokinase.

Since the establishment of the WHO First International Standard for Streptokinase and Streptodornase, 62/007, two more International Standards for streptokinase activity had been established, made using highly purified streptokinase. Currently, the streptokinase International Unit (IU) is defined by the WHO Third International Standard for Streptokinase, 00/464. A programme of work was organized to calibrate the WHO Second International Standard for Streptodornase against the WHO First International Standard for Streptokinase and Streptodornase, 62/007.

The Committee reviewed the report of a collaborative study to calibrate the activity of a candidate WHO Second International Standard for Streptodornase, 08/230, relative to the current International Standard 62/007. A total of seven laboratories returned results comprising 30 independent assays. A test method was provided and all laboratories followed this or a very similar method which
included at least a 3 dose–response curve for standard and test with two replicate readings at each dose and at least four independent assays. Data were returned and analysed at NIBSC using parallel line bioassay methods to determine the potency of the candidate International Standard, 08/230 relative to the existing WHO First International Standard for Streptokinase and Streptodornase, 62/007.

On the basis of the evidence from the study (WHO/BS/09.2112), the Committee established preparation 08/230 as the WHO Second International Standard for Streptodornase with a potency of 3200 IU per ampoule.

**Unfractionated heparin**

The Fifth International Standard for Unfractionated Heparin, 97/578 was established in 1998 by calibration against the Fourth International Standard. This standard had been widely used for calibration of clinical products and controls for diagnostic kits for heparin. Due to the depletion of stock, a replacement for 97/578 was required.

Thirty-three laboratories from 18 countries contributed data obtained from 12 different assay methods to value assign the Sixth International Standard for Unfractionated Heparin against the Fifth International Standard for Unfractionated Heparin, 97/578. Six candidates were included in the study (WHO/BS/09.2124). One candidate, 07/328, gave the lowest range of intra-laboratory variation (%GCV: 2.2–6.4) for the different assay methods and the lowest inter-laboratory variation by all methods (%GCV = 3.6) and was proposed as the replacement International Standard.

The Committee also noted that, because of recent events involving contaminated heparin, heparin manufacturers had been encouraged by regulatory authorities to replace clotting assays that can be influenced by contaminants and process-related impurities with methods that are specific for heparin, for example, anti-IIa and anti-Xa assays using purified antithrombin, for measurement of heparin potency. This study also allowed an examination of the robustness of these chromogenic methods.

This collaborative study also served to harmonize the International Unit and the United States Pharmacopeia (USP) unit for unfractionated heparin. A new (USP) Heparin Sodium Reference Standard (RS) Lot F for assay had been selected and value assigned by sub-group analysis of data obtained using the proposed new USP monograph potency method (anti-factor IIa antithrombin dependent assay; aIIa:AT). Nine participants carried out this assay. Candidate preparation 07/330 gave low intra- and inter-laboratory variation for the USP anti-IIa chromogenic assay and it also gave the best agreement of potency estimates by the three present and future USP monograph methods.

Candidate 07/330 was accepted by the USP as the USP heparin standard for assay, Lot F, with an assigned value of 2144 USP units/ampoule. As both the Sixth International Standard for Unfractionated Heparin and the USP heparin standard
for assay, Lot F, are traceable and value-assigned against the Fifth International Standard for Unfractionated Heparin, the long-standing disparity between the USP unit and the International Unit for Unfractionated Heparin was resolved.

The Committee established preparation 07/328 as the WHO Sixth International Standard for Unfractionated Heparin with an assigned value of 2145 IU/ampoule.
International reference materials – cytokines, growth factors and endocrinological substances

All reference materials established at the meeting are listed in Annex 5.

Chorionic gonadotrophin, human

At its fifty-seventh meeting, the Committee recognized the need to replace the Fourth International Standard for Chorionic Gonadotrophin, human (CG), stocks of which were almost exhausted. The standard continued to be used for controlling the quality and potency of therapeutic preparations of CG and in calibration of assays for its use for diagnosis of pregnancy. The candidate material, a highly purified preparation of urinary CG, was filled at NIBSC into ampoules in a single run and then freeze-dried. For the proposed standard (coded 07/364), the coefficient of variation for the fill was 0.10% and the residual moisture was determined to be 2.49%. The ampoules were stored at −20 °C. Stability studies had established that there was no significant loss of potency determined by immunoassay and by bioassay after storage for 6 months at up to +45 °C. The collaborative study (WHO/BS/09.2107) was performed by 19 laboratories in 11 countries. The candidate material and a candidate preparation of CG, human, recombinant were assayed by bioassay (five laboratories) and immunoassay (14 laboratories) against the existing Fourth International Standard.

On the basis of the results of the collaborative study, the Committee adopted the preparation coded 07/364 as the Fifth International Standard for Chorionic Gonadotrophin, human, and assigned a content of 179 IU per ampoule to it for the calibration of immunoassays, and a content of 162 IU per ampoule for calibration of bioassays. For the purposes of calibration of immunoassays in molar units for diagnostic purposes, the Committee also assigned a content of 0.39 nmol per ampoule.

Parathyroid hormone, 1-84, human recombinant

The Committee was reminded that, at its fifty-eighth meeting, it had accepted the need for a reference preparation of parathyroid hormone (PTH) to calibrate PTH immunoassays and the quality and potency of PTH used therapeutically. A candidate preparation of the hormone, containing 84 amino acids (PTH 1-84), obtained by recombinant methods, had been supplied to NIBSC and filled in a single run into ampoules. The ampoules were stored at −20 °C. The residual moisture content was determined to be 0.43%. Stability studies by immunoassay and by high-performance liquid chromatography (HPLC) for different periods of time up to 40 months at up to +45 °C indicated slight degradation as shown by the results of immunoassay, but not by those of HPLC. The estimated loss of activity...
nevertheless indicates that the preparation will be suitable to serve as a standard for at least 10 years when stored at −20 °C. The collaborative study (WHO/ BS/09.2115) was performed by 13 laboratories in five countries, seven of which used immunoassay, one HPLC, and five amino acid analysis.

On the basis of the results of the collaborative study, the Committee adopted the preparation coded 95/646 as the First International Standard for Parathyroid Hormone 1-84 human, recombinant, and assigned a content of 100 micrograms per ampoule to it.
International reference materials – diagnostic reagents

All reference materials established at the meeting are listed in Annex 5.

HIV-2 RNA

The second human acquired immunodeficiency syndrome (AIDS) virus HIV-2 was first isolated in 1986 from patients with AIDS in West Africa. HIV-2 was subsequently shown to be a diverse group of viruses genetically related to, and thought to be derived from, the simian immunodeficiency virus (SIV) from sooty mangabeys (Cercocebus atys). The HIV-2 group of viruses is not detected (or is poorly detected) by most commercially available HIV-1 NATs. NATs may be based on either target or signal amplification. The polymerase chain reaction (PCR) is the best-known example of target amplification assays involving thermal cycling of DNA in the presence of heat-resistant polymerases such as Taq and which results in the generation of millions of copies of the DNA sequence. DNA primers are used to amplify various viral genes or regions in env, gag, pol, and may be based on real-time technology, single or nested PCR. Other target amplification assays may be based on isothermal methods such as nucleic acid sequence-based amplification (NASBA) and transcription-mediated amplification (TMA), which are primer-dependent techniques that can be used for the continuous amplification of nucleic acids in a single mixture at one temperature. Signal amplification assays are those in which the signal is amplified, such as in the branched DNA (bDNA) assay.

Ranges of NAT are available for the amplification of HIV using all of the technologies referred to above. However, most were designed for HIV-1 and either do not amplify HIV-2 or only amplify it poorly. In recent years there has been a move to improve HIV-1 NAT to detect a wider range of subtypes, recombinants and diverse groups including, in some cases, HIV-2, as well as to enable the detection of multiple viral markers in a single assay run. Relatively few assays specific for HIV-2 had been developed although a number of such assays are in use in reference, research and other laboratories with a specific interest in HIV-2 and related viruses such as SIV.

The Committee reviewed the results of a study (WHO/BS/09.2118) designed to establish an International Standard for HIV-2 RNA that would help in the standardization of HIV-2 NAT and aid the comparison of results derived from such assays. This reagent was anticipated to be of value to kit developers, manufacturers, users, evaluators, regulators, and reference and research laboratories.

The candidate standards consisted of HIV-2 RNA in the form of heat-treated virus culture supernatant diluted in pooled human plasma (citrated). Based on experience of developing standards for other blood-transmitted viruses such as HIV-1, Hepatitis C Virus (HCV) and Hepatitis B Virus (HBV), a whole
microorganism diluted in pooled human plasma is a reasonably close homologue to the samples that are routinely used in NAT (human plasma).

Twenty-nine laboratories from 16 countries participated in a collaborative study to evaluate the HIV-2 RNA candidate standards for use with NAT. The candidate standards consisted of two HIV-2 genotype A samples, HIV-2 CAM-2 and HIV-2 ROD. Each laboratory assayed all the candidates on at least three separate occasions and the data were collated and analysed at NIBSC. Of the datasets returned, the majority were from qualitative assays. All assays detected both candidate standards with the exception of one quantitative commercial assay, Nuclisens Easy Q, which was designed primarily for HIV-1 detection; this assay did not detect HIV-2 CAM-2 but showed good detection of HIV-2 ROD. This highlighted possible cross-reactivity with HIV-2 ROD with some NAT primer/probe combinations. As a result, it was proposed that the HIV-2 CAM-2 material be established as the First International Standard for HIV-2 RNA.

The Committee agreed that HIV-2 CAM-2 (NIBSC Code 08/150) be established as the First International Standard for HIV-2 RNA and that it should be assigned a unitage of 1000 IU/ml (instead of 10 000 IU/ml as in the initial proposal) to avoid a situation in which the copy number is below the IU. The Committee also noted that the current standard represents only one subtype of HIV-2, i.e. subtype A which is the predominant strain (and also most easily grown in culture and for which the full-length sequence has been published). Future efforts should include development of an additional panel for other major subtypes of HIV-2, if possible.

**Hepatitis B virus DNA**

Hepatitis B is a potentially life-threatening liver infection caused by hepatitis B virus (HBV). It is a major global health problem and the most prevalent cause of liver cirrhosis and hepatocellular cancer. The virus is preferentially transmitted through contact with the blood or other body fluids of an infected person. Sensitive screening and accurate diagnostic assays play a crucial role in the prevention and in the management of the disease. The current WHO International Standard materials for HBV DNA and Hepatitis B surface antigen (HBsAg) were generated from HBV genotype A2/HBsAg subtype adw2. These materials have been widely used for standardization of diagnostic assays and for traceability of test results. This HBV genotype is mainly prevalent in Western Europe and in North America and represents only about 1% of the worldwide HBV-infected population. The majority of the HBV-infected people living in or coming from the Mediterranean area, Africa and Asia have the genotypes A1, B, C, D, and E, whereas F and H originate from the Americas. The origin of genotype G had not yet been clarified.

During a WHO Consultation on “Global measurement standards and their use in the in vitro biological diagnostic field”, held in June 2004, concern
was raised that HBsAg test kits and NAT test kits might be less efficient for some HBV genotypes, other than A2, represented by the current International Standard preparations. The Committee had therefore previously agreed to projects to evaluate WHO International Biological Reference Preparations for HBV DNA and for HBsAg representing different genotypes of HBV.

The Committee reviewed the results of a collaborative study (WHO/BS/09.2121) to evaluate a panel of lyophilized plasma samples containing different genotypes of HBV for use in NAT-based assays. The HBV genotype panel (PEI code number 5086/08) comprised 15 different members, which represent genotypes A (3), B (3), C (3), D (3), E (1), F (1), and G (1). Each laboratory analysed the panel samples in parallel to the Second WHO International Standard for HBV DNA (NIBSC code 97/750) representing HBV genotype A2. The study was performed on three separate occasions using quantitative NATs, or on four separate occasions using qualitative NATs. The data were collated and analysed at the PEI.

Seventeen laboratories from 12 countries participated in the study. A total of 19 sets of data were returned; 16 from quantitative NAT assays and two from qualitative NAT assays. One laboratory performed sequence and genotype analysis. The majority of NAT assays used were commercially available and based on real-time PCR. The results showed that the genotypes A–G were detected consistently by the majority of participants, although a small number of assays detected genotypes F and G less efficiently, or not at all. Only a few genotype B, C, and E samples were underquantified by the two methods used. The finding that some NAT assays had reduced detection efficiency with some of the non-A2 genotypes proved the necessity for a well-characterized genotype panel in addition to the WHO International Standard.

The Committee agreed to establish the preparations coded PEI 5086/08 as the First International Reference Panel for HBV Genotypes for NAT-based assays. No unitage was assigned to the individual panel members. The Committee also requested stability data on the panel to be presented at a future meeting.

**Parvovirus B19 DNA**

The Committee was informed that, at one time, it was believed that the genome of parvovirus B19 (B19V) was highly conserved. However, in recent years, three distinct virus genotypes and sub-genotypes had been identified and they can vary by up to 15% in nucleotide identity. Based upon sequence analysis and biological properties of these variant viruses, the Parvovirus Group of the International Committee on Taxonomy of Viruses had classified prototype representatives of the three genotypes as species of B19V. B19V is a frequent contaminant in plasma pools used in the manufacture of plasma-derived medicinal products. Screening of plasma mini-pools by NAT-based assays for B19V DNA is used to reduce the load of B19V in manufacturing pools.
The First International Standard for B19V DNA for nucleic acid amplification (NAT)-based assays (99/800) was established in 2000 and replaced by the Second International Standard for B19V DNA in 2008. The availability of the International Standard for B19V had facilitated the introduction or implementation of regulatory recommendations or requirements to improve the safety and quality of plasma-derived medicinal products, particularly solvent–detergent treated pooled plasma for which there are limited virus reduction steps during manufacture. In Europe, regulatory requirements specify that plasma pools used in the production of anti-D immunoglobulin products and plasma treated for virus inactivation are tested for levels of B19V DNA (European Pharmacopoeia monographs 0557, 1527 and 1646). Such plasma pools must not exceed a threshold concentration of 10 IU/μl for B19V DNA, as defined by the WHO International Standard. All three genotypes of B19V should be detected by NAT procedures. However, some assays for B19V DNA do not detect these variant viruses, and, as a result, some plasma pools have been contaminated with an excessive level of B19V due to assay failures. Indeed external quality assessment programmes have demonstrated that there are problems with the detection of genotypes 2 and 3 B19V by some public quality control laboratories and plasma testing organizations. Different genotypes of B19V may also be found in the clinical setting; hence, to obtain an accurate diagnosis of B19V infection, different genotypes of B19V should be detected. Clinical cases of B19V may be misdiagnosed due to false-negative results because of the inability of NAT assays to detect different B19V genotypes.

The Committee reviewed the results of a study (WHO/BS/09.2122) to evaluate a panel of plasma samples containing the three main genotypes of B19V. The panel of samples represented genotypes 1, 2, 3a B19V and a negative plasma control. Each laboratory assayed the panel members concurrently with the Second WHO International Standard for B19V DNA (NIBSC code 99/802) on four separate occasions and the data were collated and analysed at NIBSC. Thirty-five laboratories from 13 different countries participated in the study. A total of 44 sets of data were returned; 34 from quantitative assays and 10 from qualitative assays. The majority of assays used were in-house and based on real-time PCR. The results showed that all three genotypes were detected consistently by the majority of participants, although a very small number of assays detected genotypes 2 and 3 less efficiently or not at all. Real-time stability studies had indicated that the panel of B19V samples was very stable under normal conditions of storage, i.e. at −70 °C or below, and was therefore suitable for long-term use. The 4-member panel was intended for use in evaluating the ability of assays to detect different B19V genotypes and to confirm absence of cross-reactivity with pooled human plasma that is negative for B19V DNA.

The Committee recommended that the panel be established as the First International Reference Panel for Parvovirus B19 DNA Genotypes. The
Committee noted that the panel was not intended to replace the International Standard for B19V DNA. No unitage was assigned to individual panel members since the applicability of using a genotype 1 virus (i.e. the International Standard) to calibrate the genotype 2 and 3 viruses had not been determined. The B19V DNA negative plasma member was included as a control for assay specificity.

**Thromboplastin, human, recombinant plain**

According to the recommendation issued by WHO, thromboplastins used in the prothrombin time (PT) test for the laboratory control of oral anticoagulant treatment must be calibrated against International Standards to determine the international sensitivity index (ISI) necessary to convert PT results into the international normalized ratio (INR).

The First International Standard, coded 67/40, was a human brain extract to which adsorbed bovine plasma was added to create a so-called combined reagent. In 1984, 67/40 was replaced by BCT/253, a human brain extract (plain reagent). This was in turn replaced in 1996 by rTF/95 (human recombinant, plain). Stocks of rTF/95, the WHO International Standard, had become limited and needed to be replaced to maintain continuity of the calibration of thromboplastins.

The Committee reviewed the results of an international collaborative study (WHO/BS/09.2125) organized under the auspices of the SSC of the ISTH for the calibration of a replacement candidate.

Two human thromboplastin preparations, coded 07/314 and 08/144, were evaluated as potential replacement materials. The study involved 21 laboratories from 13 countries from Asia, Europe, North America and South America. The calibration was performed against the existing WHO International Standards, i.e. rTF/95 and RBT/05. The two candidates were compared based on predetermined criteria which included:

- the within-laboratory precision of calibration, assessed as the coefficient of variation (CV) for the estimation of the slope;
- the between-laboratory precision of the calibration, assessed as the CV of the international sensitivity index (ISI); and
- the conformity to the calibration model, assessed as the percentage of calibrations with important deviations of normals from patients line, according to the WHO guidelines.

Of the two candidates, preparation 08/144 gave the lowest intra- and inter-laboratory variation of the ISI, and also the lowest number of calibrations with important deviations from the WHO calibration model. Stability of the candidate reference preparations was assessed through an accelerated degradation study after storage of ampoules at elevated temperatures (+4 °C, +30 °C, +37 °C, +45 °C)
for different time intervals. For both candidates, storage at 30 °C or higher did slightly affect the ISI value. No significant change, however, was observed after storage at 4 °C, suggesting that both preparations display suitable stability when stored at –20 °C.

On the basis of the results of the collaborative study, the Committee adopted the preparation coded 08/144 as the WHO Fourth International Standard for Thromboplastin, human, recombinant, plain, with an assigned ISI value of 1.082. The Committee also requested that real-time stability monitoring data be reported at a future meeting.

**Soluble transferrin receptor**

Raised serum transferrin receptor (sTfR) concentration is a marker of iron deficiency and a WHO/CDC Technical Consultation on assessment of iron status at population level (2004) had concluded that measurement of both serum ferritin and sTfR provided the best approach for estimating the iron status of populations.

There were several manufacturers of immunoassays for sTfR, employing different local standards calibrated in different units (mg/l or nmol/l). There was thus an urgent need for an international reference material to standardize sTfR assays (there was already an International Standard for ferritin), and the Committee had previously endorsed a project for the production of a standard for sTfR, with support from the Global Alliance for Improved Nutrition (GAIN). Owing to the difficulties in purifying sufficient quantities of sTfR for use as a WHO Reference Reagent, it was decided to evaluate a recombinant soluble transferrin receptor (rsTfR) to determine its suitability for this use. The rsTfR is just a few amino acids shorter than circulating serum TfR. A trial collaborative study was conducted in 2007 in which manufacturers were invited to assay lyophilized rsTfR preparations. At a meeting convened at NIBSC with the manufacturers in October 2007, following the trial, it was agreed that the rsTfR was potentially suitable as a standard provided it could be characterized, had acceptable purity, was stable after lyophilization, and showed parallel dose–response curves in all commercially available assays. A larger, definitive fill of rsTfR in an sTfR-depleted serum matrix was therefore carried out in 2008 and subjected to another international collaborative study.

The Committee evaluated the results of the study (WHO/BS/09.2104). Seven laboratories located in Europe and the USA participated in the collaborative study. The laboratories consisted of six kit manufacturers and a health protection laboratory. The study confirmed that assays of sTfR used a variety of TfR standards calibrated in different units, and found that there was poor agreement between the assay methods used in the study even when values were expressed in mg/l units of measurement. The rsTfR preparation coded 07/202 demonstrated acceptable overall parallelism to the manufacturers’ in-house standards and to
three serum samples included in the study. The inclusion of these serum samples indicated that the use of rsTfR preparation 07/202 as a Reference Reagent would significantly reduce inter-method variability.

On the basis of the results of the collaborative study, the Committee adopted the preparation coded 07/202 as a WHO Reference Reagent for soluble transferrin receptor with assigned values of 21.7 mg/l and 303 nmol/l (when reconstituted with 0.50 ml distilled or deionized water). These values apply to free rsTfR monomer. The Reference Reagent was intended to be used to standardize immunoassays for sTfR.

**Prader-Willi and Angelman syndromes**

Prader-Willi (PWS; OMIM #176270) and Angelman (AS; OMIM #105830) syndromes are clinically distinct genetic disorders, both mapping to chromosome 15q11-q13.

PWS is the most common form of obesity caused by a genetic syndrome and is characterized by muscular hypotonia (“floppy baby”), a failure to thrive during infancy, mental retardation, hypogonadism and hyperphagia (excessive appetite or eating) leading to rapid weight gain between one and six years of age. Clinical diagnosis is often difficult due to the relatively non-specific findings, particularly in infancy. Moreover, there is clinical overlap with numerous other disorders including fragile X syndrome, Bardet-Biedl syndrome and Cohen syndrome, compounding the difficulty of clinical diagnosis. The incidence of PWS is reported as 1 in 25 000, but this is considered by some authorities to be an underestimate. As with most genetic disorders, PWS is equally prevalent across all populations.

AS is characterized by severe mental retardation, movement or balance disorder, characteristic abnormal behaviours (including apparent happy demeanour and hand flapping movements) and severe speech and language limitations. Clinical diagnosis may be delayed since the unique features of AS may not become apparent for several years. Additionally, there are many other disorders which clinically overlap with AS including cerebral palsy, Rett syndrome and idiopathic static encephalopathy, which can lead to an incorrect clinical diagnosis of AS. The incidence of AS is reported as between 1 in 12 000 and 1 in 20 000, but this is also considered by some authorities to be an underestimate due to the difficulty of making a clinical diagnosis at an early age. AS is also not restricted to particular populations.

Genetic testing for PWS and AS has become the standard diagnostic method because clinical criteria, although defined, are not always specific. Furthermore, the risk of a subsequent child being affected by the same syndrome, for parents of a child affected with PWS or AS, can only be determined based on the results of the genetic testing since the genetics of these syndromes are complex. With confirmed genetic diagnosis, treatment of an affected individual
can be begun earlier in life and the condition continuously managed to enhance quality of life and even prevent early death.

Genetic testing for PWS and AS is widespread, but due to the genetic complexity of the disorders and the diversity of techniques available for genetic diagnosis there is a need for widely available genetic reference materials to ensure a correct and consistent diagnostic outcome. Hence, although both disorders are relatively uncommon, the need for confirmatory and accurate genetic diagnostic testing is paramount.

The Committee was informed that no internationally certified genetic reference materials were available for the clinical diagnosis of PWS or AS. Most laboratories used patient-derived DNA samples, which had been characterized in-house, as controls. If new to the field, laboratories often relied on small finite amounts of materials supplied by other diagnostic laboratories. However, a continuous supply of stable and reliable reference materials was unlikely to be guaranteed.

The Committee reviewed the results of a study a panel of genomic DNA (gDNA) materials. The gDNA was extracted from immortalized cell lines produced by Epstein–Barr Virus (EBV) transformation of lymphocytes from donors known to carry PWS or AS mutations. These materials, from consenting donors, were well characterized with confirmed mutations and, because they were obtained from cell lines, future supplies of identical materials could be ensured. Thirty-seven laboratories participated in an international collaborative study (WHO/BS/09.2105) to assess the suitability of the panel of gDNA samples. Participants evaluated the samples using their routine diagnostic methods and, where possible, against in-house controls (previously characterized patient samples) and commercial reference materials. A total of 666 tests were carried out (18 samples in each of 37 laboratories) and 38 incorrect results were reported, giving an overall error frequency of 5.71%.

Conclusions from the study indicated that all six materials were suitable for use as reference materials in the genetic diagnosis of Prader-Willi and Angelman syndromes.

On the basis of the results of the collaborative study, the Committee adopted the panel of six preparations coded 09/140 as the First International Genetic Reference Panel for Prader-Willi and Angelman Syndromes.

**Fusion gene BCR–ABL**

Chronic myeloid leukaemia (CML) represents about 15–20% of all cases of adult leukaemia and acute lymphoblastic leukaemia (ALL) accounts for approximately 80% of all childhood leukaemia cases. Nearly all cases of CML and a minority of cases of ALL are caused by a chromosome translocation t(9;22)(q34;q11), known as the Philadelphia chromosome, which fuses two genes: BCR and ABL. The BCR–ABL fusion acts as an oncogene and promotes genomic instability.
Reverse-transcription real-time quantitative PCR (RQ-PCR) is routinely used to quantify levels of BCR–ABL mRNA transcripts in peripheral blood and bone marrow samples from patients with CML. The technique can determine accurately the response to treatment and is particularly valuable for patients who have achieved complete chromosomal remission. Despite efforts to establish standardized protocols for BCR–ABL fusion transcript quantitation there is still substantial variation in the way in which RQ-PCR for BCR–ABL is carried out and in how results are reported in different laboratories worldwide. In particular, the use of different control genes for normalization of results means that there are several different units of measurement worldwide.

Recommendations for the harmonization of RQ-PCR for BCR-ABL had been made including the use of one of three control genes and, most importantly, a new international scale for BCR–ABL RQ-PCR measurements had been proposed. The concept of the international scale is similar to established procedures for other quantitative assays, for example the INR for prothrombin time. The availability of internationally accredited reference reagents should, in principle, help to make the international scale more accessible, as well as providing a more robust framework for the scale itself. This should allow better comparability between laboratories and lead to the standardization of patient assessment.

Ideally, the formulation for reference reagents should be as close as possible to the usual analyte, should cover the entire analytical process and should be applicable to methods in use throughout the world. However, it is essential that the formulation is stable over a period of several years and that it is physically possible to produce batches of sufficient size to satisfy demand over a similar period of time. It has been shown previously that good quality RNA can be extracted from freeze-dried K562 cells and therefore one possible solution is the use of freeze-dried cell line mixtures. This strategy was presented and agreed upon at a meeting of the International BCR-ABL standardization group, an informal network which meets biannually at the annual American Society of Hematology and European Hematology Association meeting and which is attended by representatives from public and private testing laboratories throughout the world.

The Committee considered the results of an international collaborative study (WHO/BS/09.2106) to assess the use of freeze-dried cell line mixtures as universal reference materials in support of the international scale. Ten laboratories participated and the results indicated that all of the materials were suitable for use as reference materials for the quantitation of BCR–ABL translocation using RQ-PCR. All of the participating laboratories agreed with the recommendation.

On the basis of the results of the collaborative study, the Committee adopted the panel coded 09/138 as the WHO First International Genetic Reference Panel for the quantitation of BCR–ABL translocation.
Proposed new reference preparation projects

Antigens and related substances

Human papillomavirus (HPV) type 6 DNA and type 11 DNA – proposed International Standards

Human papillomavirus (HPV) type 6 and type 11 are the main serotypes responsible for human genital warts. The need had been identified (WHO/BS/09.2126) for International Standards for human papillomavirus (HPV) type 6 DNA and type 11 DNA to standardize assays. The plasmid preparations would be used by vaccine manufacturers, control laboratories and public health laboratories to facilitate epidemiological studies and studies on the impact of vaccines. The plasmids would be characterized and then distributed for assessment of their performance in quantitative PCR assays. The Committee accepted the proposal to prepare plasmid standards of HPV type 6 and type 11 and to conduct the collaborative study outlined.

Purified human genomic DNA

The Committee was reminded that a number of International Standards for HPV type-specific DNA had been established. The need had now been identified for a preparation of purified human genomic DNA for use as a diluent in assays of HPV standards to ensure that all dilutions employed in assays contain a suitable background level of human genomic DNA derived from an appropriate source (WHO/BS/09.2126). Provision of a common preparation for this purpose would be valuable. It had been suggested that the DNA should be derived from C33A cells but certain issues of intellectual property would require resolution and a different source may need to be found. The Committee endorsed this proposal to prepare and study the use of the proposed material.

Typhoid antibodies – proposed International Standards

Typhoid is a major cause of illness and death among children in developing countries. The former International Standard for anti-Vi serum is exhausted and the development of new conjugated vaccines under clinical trial had highlighted the need for new International Standards for anti-Vi capsule, human and for anti-Vi capsule, sheep for use in manufacture and control of vaccines and in diagnosis. Candidate material was in the process of being collected (WHO/BS/09.2126). The Committee agreed that steps should be taken to obtain appropriate material and assess its suitability for use in a collaborative study.

Antibodies (human) to influenza A virus – proposed International Standards

The Committee was reminded that an International Standard for antibodies to influenza A/H5N1 clade 1 virus had been adopted at its previous meeting.
This was not suitable for studies on antibodies to other H5 virus clades. It was proposed that new International Standards should be prepared for antibodies to influenza A/H5N1 virus, in particular to influenza A/H5N1 clade 2 virus (WHO/BS/09.2126).

In addition, the Committee was informed that many pandemic H1N1 vaccine clinical trials were taking place. Pandemic H1N1 serology assays would vary between laboratories, and it was likely that expression of results relative to a standard would reduce between-laboratory variation. NIBSC reported that suitable candidate materials for antibodies to H1N1 pandemic virus had been obtained filled and studies were under way. It was agreed that a report should be submitted as soon as possible and that adoption of the standards should be expedited between Committee meetings.

The Committee requested the Secretariat to draw up a procedure for such expedited adoption to be used in the future if required.

**Antibodies (human) to pneumococcal polysaccharides – proposed International Standard**

The first pneumococcal reference serum had been used widely in serological evaluation of candidate pneumococcal vaccines. The vaccines evaluated have ranged from those with seven components to those with 23 components. To ensure that the relationship between the clinical efficacy of the vaccines is maintained, the need had been identified for a new International Standard for antibodies (human) to pneumococcal polysaccharides. Volunteers had been vaccinated with a 23-valent vaccine: serum was collected after 4 and 8 weeks, pooled, filled at 6 ml per vial under contract and lyophilized. Approximately 15,300 vials were obtained. It was proposed to link this material to the existing serum by suitable bridging studies. The Committee endorsed this proposal (WHO/BS/09.2126).

**Mycoplasma DNA – proposed International Standard**

The need had been identified for an International Standard for NAT assays of mycoplasma DNA (WHO/BS/09.2126 Addendum 1) because of the lack of standardization of NAT assays for detection of mycoplasmas. The *European Pharmacopoeia* had introduced NAT techniques for detection of mycoplasma in veterinary products and later extension to human medicines was envisaged. However, evaluating the sensitivity of the NAT assays remains problematic without an available standard. It was proposed to obtain a culture of a single species of mycoplasma and lyophilize it. The Committee agreed that a standard is required for validation of NAT methods by manufacturers and control laboratories but recommended that more information be sought and that the project should be discussed at the next meeting of the WHO collaborating centres, taking account of other work that may be in progress.
WHO Vero reference cell bank 10-87 – proposed replacement cell bank

WHO had established a reference cell bank of Vero cells (10-87) that had been widely used as a well-characterized cell seed stock for vaccine development. The stocks of WHO Vero 10-87 cells were limited and their use had increased, so a need had been identified for a replacement of the current cell bank (WHO/BS/09.2126 Addendum 1) in order to maintain future stocks. The Committee agreed in principle to this request but asked that the WHO Group on cell substrates should investigate what was needed and report back. The Committee emphasized the importance of stipulating that the cell bank is intended for use as a cell seed and not for production of licensed products.

Anti-malaria IgG – proposed International Standard

The need had been identified for a new International Standard for anti-malaria IgG (WHO/BS/09.2126) for use in the development of malaria vaccines. Such a material would also be valuable for use as an immunoepidemiological reference material. Sera were being collected from adults in Kenya who were recovering from infection with malaria and whose blood had been appropriately screened. The Committee recognized the importance of such a standard in moving towards vaccine development and monitoring of anti-malaria public health interventions and endorsed the proposal. The Committee requested that the candidate material be designated anti-\textit{Plasmodium falciparum} IgG.

Blood products and related substances

Transfusion-relevant bacterial strain panel

The Committee endorsed a proposal (WHO/BS/09.2126) to prepare a transfusion-relevant bacterial strain panel of four frozen bacterial species (\textit{Staphylococcus epidermidis}, \textit{Streptococcus pyogenes}, \textit{Escherichia coli}, and \textit{Klebsiella pneumoniae}) as a WHO reference panel. The intended use is for the standardization of validation and assessment of methods for improvement of microbial safety of platelet concentrates (PCs). The panel members are bacterial strains selected for their ability to replicate in PCs under routine storage conditions used in transfusion medicine. The panel members would be prepared using a specially developed procedure which guarantees defined bacterial suspensions (deep frozen, ready to use, stable, shippable, defined in count of living cells). The panel was designed to allow objective validation of methods for bacterial screening as well as technologies for pathogen reduction in PCs under “real life” conditions, i.e. inoculating the PCs with a very low bacteria count (0.03 to 0.3 CFU/ml) followed by growth in the matrix. Until now, there had been no transfusion-relevant bacterial reference strains available.

The Committee requested stability data, clarification of the procedures used to prepare the batches, clarification of the issues of custodianship and
distribution of the panel should it be established, and that consideration be given to enlargement of the panel (to a minimum of 10 bacterial species).

**Fibrinogen, plasma**

Stocks of the current WHO Second International Standard (98/612) were running low and were likely to be exhausted by the end of 2011. This standard is used for measurement of fibrinogen concentration in patients’ plasma to diagnose fibrinogen deficiencies and other haemorrhagic and cardiovascular disorders. The current standard is primarily used by hospitals and by clinical laboratories. The Committee endorsed a proposal (WHO/BS/09.2126) that material for a replacement be sourced from transfusion centres in the United Kingdom and an international collaborative study be organized to evaluate the suitability of the candidate replacement.

**Fibrinogen, concentrate**

Stocks of the current WHO First International Standard (98/614) were running low and were likely to be exhausted by the end of 2011. This standard is used for the potency measurement of fibrinogen in therapeutic fibrinogen concentrates and in fibrinogen components of fibrin sealants, which are used for the treatment of fibrinogen deficiencies and in clinical surgical operations to stem bleeding, respectively. The Committee endorsed a proposal (WHO/BS/09.2126) that material for a replacement be obtained. The project would be carried out in association with the SSC of the ISTH. Small trial fills would be carried out to confirm a suitable formulation prior to the definitive fill. Calibration would be performed by clotting assays and relative to the current WHO First International Standard in an international multicentre study involving manufacturers, clinical laboratories and regulatory authorities.

**Low molecular weight (LMW) heparin**

The Committee was informed that there was approximately 4–5 years’ supply of the Second International Standard for LMW heparin in stock. There was a need to ensure the continuity of this standard by preparing a replacement. This standard is used for potency estimation of LMW heparin therapeutics as well as for calibration of reference standards for in vitro diagnostic kits. The Committee endorsed a proposal (WHO/BS/09.2126) that active pharmaceutical ingredients for LMW heparin be sourced as candidate replacement materials. Since the establishment of the First International Standard, new generations of LMW heparin were either on the market or in phase III clinical trials, thus detailed investigation would be required to ensure good comparability of all products with proposed candidate reference materials.
Cytokines, growth factors and endocrinological substances

Follicle-stimulating hormone – proposed Second International Standard

Stocks of the First International Standard for Follicle-Stimulating Hormone (FSH) were very low. The standard is required for the assignment of potency to therapeutic products for treatment of infertility. A candidate material consisting of highly purified recombinant FSH had been obtained by NIBSC from a manufacturer. The Committee agreed with the proposal (WHO/BS/00.2126) that a replacement International Standard be prepared.

Transforming Growth Factor β3 – proposed International Standard or reference reagent

Transforming growth factor β3 is widely distributed in tissues and has a variety of actions including those on cell proliferation and differentiation. A number of therapeutic uses were being investigated and the assays used for potency assignment would benefit from availability of a standard. Two candidate preparations are available, one from insect cells and the other expressed in *E. coli*. The Committee accepted the proposal (WHO/BS/09.2126) to proceed with fills and organization of a collaborative study.

Granulocyte Colony-Stimulating Factor – proposed Second International Standard

The Committee was informed that stocks of the First International Standard for Granulocyte Colony-Stimulating Factor (G-CSF) were depleted. The standard is required for the determination of potency of therapeutic products used in several indications relating to neutropaenia. A candidate material had been obtained by NIBSC and included in the collaborative study on which the current standard was established. Its stability would be examined. Two other candidates were available. The Committee approved the proposal (WHO/BS/09.2126) that a replacement International Standard be prepared.

Insulin, human, recombinant – proposed International Standard

WHO had received a request (WHO/BS/09.2126 Add. 1) to establish a new International Standard for human insulin of recombinant origin to be used as a primary chemical standard for calibration of pharmacopoeial and manufacturers’ standards. The request raised many critical issues that would require detailed consideration by suitable experts. The Committee considered that its expertise would need to be supplemented by that of the Expert Committee on Specifications for Pharmaceutical Preparations, which is responsible for WHO chemical reference standards. The Committee requested that a detailed study plan be disseminated and discussed by a broad group including regulators and experts in establishing chemical reference materials.
Diagnostic reagents

HIV-1 RNA for NAT-based assays

The Committee agreed with a proposal (WHO/BS/09.2126) to evaluate replacements for the Second International Standard for HIV-1 RNA, which was established by the Committee in 2005. Stocks had fallen to <1000 and it was predicted that there was only enough stock remaining to last for about 3.5 years. It was proposed to use HIV-1 genotype B strain, as was used to manufacture the Second International Standard, and to dilute the virus in pooled human plasma. The virus would be heat-inactivated before freeze-drying. A wide range of assays of both qualitative and quantitative assay formats would be used in the collaborative study. End-point estimates would be obtained from qualitative assays and viral loads from quantitative assays. It was anticipated that the new reference preparation would be used to calibrate secondary standards and would be used by a range of laboratories including kit manufacturers, blood fractionators, reference laboratories, and national control laboratories.

Antibodies to human T-lymphotropic virus type 1 (HTLV-1) and type 2 (HTLV-2)

HTLV-1 and HTLV-2 are infectious agents that pose significant risks to the blood supplies in specific areas, including Africa (HTLV-1 and HTLV-2), South America (HTLV-2), the Caribbean (HTLV-1), and Japan (HTLV-1). The lack of reference panels hinders the evaluation of new technologies that would aid in the screening for these agents. In addition, some HTLV-2 subtypes may escape detection by currently available technology. The Committee agreed with a proposal (WHO/BS/09.2126) to develop an anti-HTLV Reference Panel for use as a reference standard for the development or use of assays for the detection of antibodies to HTLV-1 and HTLV-2 in blood and blood products. Such a material would be used by blood screening and diagnostic test developers, blood banks, hospitals and other establishments performing HTLV serology.

Dengue virus RNA for NAT-based assay reference panel

Dengue is a bloodborne pathogen and the risk of transmission by blood transfusion has already been documented. The availability of a Dengue RNA reference panel would facilitate the development and the quality control of new assays for detection of dengue for both diagnostic purposes and blood screening. Such assays would significantly improve patient care and the safety of the blood supply worldwide.

The Committee endorsed a proposal (WHO/BS/09.2126) to develop a Dengue 1-4 NAT reference panel. The intended use of such a standard would be in the development of assays for the diagnosis and detection of dengue in blood and blood products. It may also be used as reference control to evaluate performance
of assays for the detection of dengue. Potential users would be blood screening and diagnostic test developers, blood banks, hospitals and other establishments performing dengue testing. The standard may serve for standardization of both qualitative and quantitative diagnostic kits by manufacturers and for calibration of secondary standards.

**West Nile Virus RNA for NAT-based assays**

West Nile virus (WNV) is a bloodborne pathogen that is known to be transmitted by blood transfusion. The availability of an International Standard for WNV RNA would facilitate the development and quality control of new NAT assays for use in blood donor screening and in medical diagnostic testing. Such assays would significantly improve the safety of the blood supply and patient management worldwide. The Committee endorsed a proposal (WHO/BS/09.2126 Add.1) to develop such a standard from WNV viral isolates from infected human plasma specimens, and characterized by gene sequencing, viral load and infectivity titre measurements. A reference material for WNV RNA would be used as a standard for the development of assays for the detection of WNV infection in blood donors. It may also be used as a reference reagent to evaluate the performance of assays for detection of WNV viral RNA. Users would include blood donor screening and diagnostic test developers, blood banks, hospitals, clinical laboratories and other establishments performing WNV testing.

**Hepatitis C virus (HCV) core antigen**

The Committee was informed that an HCV core antigen standard would be very useful for estimating the analytical sensitivity of HCV core antigen (Ag) and HCV Ag/antibody combination assays. It may serve for standardization of both qualitative and quantitative diagnostic kits by manufacturers and for calibration of secondary standards. A proposal (WHO/BS/09.2126) to develop such a standard from HCV antigen-positive human plasma was endorsed.

**Hepatitis D virus RNA for NAT-based assays**

The hepatitis D virus (HDV) is a defective single-stranded RNA virus that requires the helper function of hepatitis B virus (HBV) to replicate. The envelope of HDV particles contains the hepatitis B surface antigen (HBsAg). HDV can be acquired either as a coinfection with HBV or as a superinfection of people with chronic HBV. HDV genotype 1 (HDV-1) is the predominant form worldwide, and is associated with a broad spectrum of chronic HDV disease. The Committee was informed that the methods of choice for the diagnosis of ongoing HDV infection were NAT assays. A major problem in the treatment of chronic hepatitis D is monitoring the response to therapy, because no standardized assays for the assessment of HDV
viraemia were available. Standardization in the performance of HDV diagnostic assays, including NAT assays, and the development of standardized treatment models had been hampered by the lack of an International Standard for HDV. Furthermore, standardized assays would improve HDV screening in endemic areas to obtain more epidemiological data.

The Committee agreed with a proposal (WHO/BS/09.2126) to develop such a standard from HDV-1RNA-positive plasma samples.

**Hepatitis E virus (HEV) RNA for NAT-based assays**

The Committee was informed that hepatitis E virus (HEV) is a major public health concern, responsible for >50% of acute viral hepatitis cases in endemic areas (Africa, Asia and Central America) where sanitation is poor. High mortality rates of up to 25% occur in pregnant women and individuals with underlying liver disease. In industrialized countries HEV infection may be linked to travel to endemic areas. Autochthonous cases, however, are increasing, with zoonotic transmission from pigs and other species. HEV viraemia and faecal shedding occur several weeks before the development of anti-HEV IgM and IgG. It is recognized that HEV diagnostic testing, including (NAT), is important in patients in whom other causes of acute hepatitis have been excluded. Transmission of HEV through transfusions occurs and the virus is relatively resistant to viral inactivation and removal procedures. Consequently, NAT screening had been proposed for certain classes of plasma-derived products with limited virus reduction steps. Vaccines against HEV were under development.

Several high-titre human plasma samples viraemic for HEV, and bile specimens from infected pigs had been obtained. An initial study was proposed comprising a panel of HEV-positive samples diluted in human plasma. The panel would contain different viral strains covering a range of concentrations. The results of this initial evaluation panel would provide information concerning assay performance and for determining the most suitable virus strain for development into a standard. The Committee endorsed the proposed project (WHO/BS/09.2126).

**Toxoplasma gondii DNA for NAT-based assays**

Toxoplasmosis is an infectious disease caused by the parasitic protozoan *Toxoplasma gondii*. It is endemic worldwide and, depending on geographical location, 15–90% of the human population are asymptptomatically infected. Routine diagnosis is carried out by serology. However, the parasite is a major opportunistic pathogen of immunocompromised patients. Also, if pregnant women develop a primary infection it can be transmitted to the fetus and cause serious damage. For these two subpopulations, a rapid and accurate diagnosis is required so that suitable treatment can be provided. Serological diagnosis is not
always reliable, as reactivation is not always accompanied by changes in antibody levels. The presence of IgM does not necessarily indicate recent infection either. For these reasons PCR is frequently used in the diagnosis of *T. gondii* in these at-risk patients. PCR had been found to be a sensitive, specific, and rapid method for the detection of *T. gondii*. However, no international reference materials existed for the detection of *T. gondii* by NAT assays.

The Committee endorsed a proposal (WHO/BS/09.2126) to develop a standard that would be used to aid in the detection of *T. gondii* in immunocompromised patients and in pregnant women by hospital and reference laboratories. The Committee noted that the European Food Standards Agency had recently expressed the need for standardization of assays for *T. gondii*.

**Anti-toxoplasma antibody IgM, human**

The Committee was reminded of the need for calibration of diagnostic reagents and tests for toxoplasmosis. It endorsed a proposal (WHO/BS/09.2126) to evaluate a candidate IgM reference material to supplement existing WHO International Standard 01/600 (anti-Toxoplasma IgG) and replace a previous WHO International Standard (TOXM; anti-Toxoplasma Ig), which was now exhausted. IgG/IgM-positive sera would be collected from volunteers by a reference laboratory linked to a hospital as previously done for 01/600. It was proposed to remove IgG from the material using protG, thus creating an IgM-only standard.

**BK virus DNA for NAT-based assays**

BK virus (BKV) is a ubiquitous polyomavirus. Whereas primary infection is usually asymptomatic, the virus establishes lifelong latency and may reactivate and cause disease in the absence of effective immune control. BKV is responsible for severe nephropathy (polyomavirus-associated nephropathy, or PVAN) in kidney transplant recipients. The incidence of PVAN had increased with the widespread use of third-generation immunosuppressive agents (tacrolimus and mycophenolate mofetil (MMF)). BK virus has also been associated with haemorrhagic cystitis following haematopoietic stem cell transplantation.

BKV DNA in plasma is recognized as a surrogate marker for the diagnosis of PVAN. Viral load measurements using NAT are used to guide the initiation of and to monitor the response to therapy. The results of recent worldwide external quality assessment (EQA) proficiency programmes for BKV NAT assays had demonstrated a significant degree of variation in both the detection and quantification of BK virus, highlighting the need for standardization of these assays. Calibration of secondary reference reagents used in routine diagnostic NAT assays, against an internationally accepted primary reference would enable more meaningful comparisons of assay results and the development of uniform therapeutic strategies.
The Committee agreed with a proposal (WHO/BS/09.2126) to develop such a standard.

**JC virus DNA for NAT-based assays**

JC virus (JCV) is a ubiquitous polyomavirus. While primary infection is usually asymptomatic, the virus establishes lifelong latency and may reactivate and cause disease in the absence of effective immune control. JCV is the pathogen responsible for progressive multifocal leukoencephalopathy (PML); a severe, usually fatal, demyelinating disease of the central nervous system which affects people with HIV/AIDS and transplant recipients. PML had also recently emerged in multiple sclerosis patients treated with monoclonal antibodies. Diagnosis of JCV relies on NAT and, increasingly, viral load measurements are used to monitor the response to therapy. The results of recent worldwide EQA proficiency programmes for JCV NAT assays demonstrated a significant degree of variation in both detection and quantification of JCV, and poor assay sensitivity, highlighting the need for standardization of these assays. Calibration of secondary reference reagents used in routine diagnostic NAT assays, against an internationally accepted primary reference would enable more meaningful comparisons of assay results and the development of uniform therapeutic strategies.

The Committee agreed with a proposal (WHO/BS/09.2126) to develop such a standard.

**Apolipoprotein B reagent**

The Committee had recognized in 1993 the need for an international standard for apolipoprotein B for the standardization and calibration verification of immunoassays used for assessing cardiovascular disease (CVD) risk and monitoring disease status.

Evaluation of the First International Reference Reagent for apolipoprotein B, coded SP3-07, had been carried out under the auspices of the International Federation of Clinical Chemistry Committee on Apolipoproteins. The stock of SP3-07 had become depleted. Therefore it was necessary to prepare a replacement lot of the International Standard.

The Committee considered a proposal (WHO/BS/09.2126 Add.1) to prepare a new lot of International Standard, to be coded as SP3-08, for use as a standard for the verification of calibration and standardization of assays for the measurement of apolipoprotein B as a risk indicator for CVD. It may also be used as a reference reagent to evaluate the performance of assays for the measurement of apolipoprotein B. Users would be manufacturers of in vitro diagnostic assays, clinical laboratories and other establishments performing apolipoprotein B testing.

The proposal was endorsed by the Committee.
Antibodies to dsDNA (human serum)

Systemic lupus erythematosus (SLE) is a chronic autoimmune connective tissue disease and the antibodies against dsDNA are highly specific for SLE, occurring in ~70% of cases (compared to only ~0.5% of people without SLE). The WHO International Standard (W1065/Wo80) for anti-dsDNA was established in 1985 and its IU assignment used to standardize commercial ELISA kits; it is also used to validate immunofluoresence tests.

When WHO International Standard preparations were transferred from Sanquin to NIBSC in 2007, it was discovered that stocks of this preparation were completely exhausted. The Committee endorsed a proposal (WHO/BS/09.2126) to establish collaborations with appropriate clinicians and scientists working in the field, as well as kit manufacturers, to try to source suitable bulk for filling and to carry out a collaborative study and assign unitage based on kit standards calibrated against the First International Standard.
Ongoing stability monitoring for WHO international standards

Prediction of stability is normally done at the time of adoption of a reference material. Subsequent monitoring of selected established standards had been requested but there was a need for guidance on how to report such data to the Committee. A summary table was proposed to the Committee with examples of its completion for the Second International Standard for somatropin (recombinant human growth hormone) and for the International Standard for Tetanus Toxoid for use in Flocculation Test. On the basis of these examples, the proposed format was accepted by the Committee for future formal reporting of post-establishment stability data.
Annex 1

Recommendations, Guidelines and other documents for 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 Organization1 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
1211 Geneva 27
Switzerland

1 Abbreviated in the following pages as TRS.
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Annex 2

Guidelines on evaluation of similar biotherapeutic products (SBPs)

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1. Introduction

Biotherapeutic products (biotherapeutics) have a successful record in treating many life-threatening and chronic diseases. However, their cost has often been high, thereby limiting their accessibility to patients, particularly in developing countries. Recently, the expiry of patents and/or data protection for the first major group of originator’s biotherapeutics has ushered in an era of products that are designed to be “similar” to a licensed originator product. These products rely for their licensing partly on prior information regarding safety and efficacy obtained with the originator products. The clinical experience and established safety profile of the originator products should contribute to the development of similar biotherapeutic products (SBPs). A variety of terms, such as “biosimilar products”, “follow-on protein products” and “subsequent-entry biologics” have been coined to describe these products.

The term “generic” medicine is used to describe chemical, small-molecule medicinal products that are structurally and therapeutically equivalent to an originator product whose patent and/or data protection period has expired. Demonstration of bioequivalence of the generic medicine to a reference product is usually appropriate and sufficient proof of therapeutic equivalence between the two. However, the approach established for generic medicines is not suitable for the development, evaluation and licensing of SBPs since biotherapeutics consist of relatively large and complex proteins that are difficult to characterize. The clinical performance of biotherapeutics can also be much influenced by the manufacturing process and some clinical studies will also be required to support the safety and efficacy of a SBP.

As part of its mandate for assuring global quality, safety and efficacy of biotherapeutics, WHO provides globally accepted norms and standards for the evaluation of these products (1, 2). Written standards established through the Expert Committee on Biological Standardization (ECBS) serve as a basis for setting national requirements for production, quality control and overall regulation of biological medicines. In addition, International Standards for measurement are essential tools for establishing the potency of biological medicines worldwide (3); they are often used as primary standards for calibration of the secondary standards that are directly used in the biological assays.

An increasingly wide range of so-called SBPs¹ is under development or is already licensed in many countries and a need for guidelines for their evaluation and overall regulation was formally recognized by WHO in 2007 (4). This document is intended to provide guidance for the development and evaluation

¹ Not all products deemed to be SBPs will be consistent with the definition of SBPs and/or process for their evaluation described in these guidelines.
of such biotherapeutics; it should be viewed as a “living” document that will be developed further in line with advances in scientific knowledge and experience.

It is essential that the standard of evidence supporting the decisions to license SBPs be sufficient to ensure that the products meet acceptable levels of quality, safety and efficacy for public health purposes. Elaboration of the data requirements and considerations for the licensing of these products is expected to facilitate development of and worldwide access to biotherapeutics of assured quality, safety and efficacy at more affordable prices. In most cases, their authorization will be evaluated on a case-by-case basis, and the amount of data required by a national regulatory authority (NRA) may vary. However, it is expected that Guidelines on the scientific principles for evaluation of SBPs will help to harmonize the requirements worldwide and lead to easier and speedier approval and assurance of the quality, safety and efficacy of these products. It is important to note that biotherapeutics that are not shown to be similar to a reference biotherapeutic product (RBP) as indicated in these Guidelines should neither be described as “similar” nor called SBPs. Such products could be licensed through the usual processes, using more extensive nonclinical and clinical data sets or full licensing applications.

It was recognized that a number of important issues associated with the use of SBPs, including but not limited to the following, need to be defined by NRAs:

- intellectual property issues;
- interchangeability and substitution of RBP with SBP; and
- labelling and prescribing information.

For this reason, these issues are not elaborated in this document.

2. Aim

The intention of this document is to provide globally acceptable principles for licensing biotherapeutic products that are claimed to be similar to biotherapeutic products of assured quality, safety, and efficacy that have been licensed based on a full licensing dossier. On the basis of proven similarity, the licensing of a SBP will rely, in part, on nonclinical and clinical data generated with an already licensed RBP. These Guidelines can be adopted as a whole, or partially, by NRAs worldwide or used as a basis for establishing national regulatory frameworks for licensure of these products.

3. Scope

These Guidelines apply to well-established and well-characterized biotherapeutic products such as recombinant DNA-derived therapeutic proteins. Vaccines and
plasma-derived products and their recombinant analogues, for which WHO Recommendations and regulatory guidance are available elsewhere (http://www.who.int/biologicals/areas/en/), are excluded from the scope of this document.

4. Glossary

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

**Comparability exercise.** Head-to-head comparison of a biotherapeutic product with a licensed originator product with the goal of establishing similarity in quality, safety and efficacy. Products should be compared in the same study using the same procedures.

**Drug product.** A pharmaceutical product type that contains a drug substance, generally in association with excipients.

**Drug substance.** The active pharmaceutical ingredient and associated molecules that may be subsequently formulated, with excipients, to produce the drug product. It may be composed of the desired product, product-related substances, and product- and process-related impurities. It may also contain other components such as buffers.

**Equivalent.** Equal or virtually identical in the parameter of interest. Equivalent efficacy of two medicinal products means they have similar (no better and no worse) efficacy and any observed differences are of no clinical relevance.

**Generic medicine.** A generic medicine contains the same active pharmaceutical ingredient as, and is bioequivalent to, an originator (comparator) medicine. Since generic medicines are identical in the active pharmaceutical substance, dose, strength, route of administration, safety, efficacy and intended use, they can be substituted for the originator product.

**Head-to-head comparison.** Direct comparison of the properties of the SBP with the RBP in the same study.

**Immunogenicity.** The ability of a substance to trigger an immune response or reaction (e.g. development of specific antibodies, T cell response, allergic or anaphylactic reaction).

**Impurity.** Any component present in the drug substance or drug product that is not the desired product, a product-related substance, or excipient including buffer components. It may be either process- or product-related.

**Non-inferior.** Not clinically inferior to a comparator in the parameter studied. A non-inferiority clinical trial is one that has the primary objective of showing that the response to the investigational product is not clinically inferior to a comparator by a pre-specified margin.

**Originator product.** A medicine that has been licensed by the national regulatory authorities on the basis of a full registration dossier; i.e. the approved indication(s) for use were granted on the basis of full quality, efficacy and safety data.
Pharmacovigilance. The science and activities relating to the detection, assessment, understanding and prevention of adverse effects or any other drug-related problems.

Reference biotherapeutic product (RBP). A reference biotherapeutic product is used as the comparator for head-to-head comparability studies with the similar biotherapeutic product in order to show similarity in terms of quality, safety and efficacy. Only an originator product that was licensed on the basis of a full registration dossier can serve as an RBP. The term does not refer to measurement standards such as international, pharmacopoeial or national standards or reference standards.

Similarity. Absence of a relevant difference in the parameter of interest.

Similar biotherapeutic product (SBP). A biotherapeutic product that is similar in terms of quality, safety and efficacy to an already licensed reference biotherapeutic product.

Well-established biotherapeutic product. A biotherapeutic product that has been marketed for a suitable period of time with a proven quality, efficacy and safety.

5. Scientific considerations and concept for licensing SBPs

The regulatory framework for the licensing of generic medicines is well established in most countries. Demonstration of structural sameness and bioequivalence of the generic medicine to the reference product is usually sufficient for therapeutic equivalence between the generic and reference product to be inferred. However, the generic approach is not suitable for the licensing of SBPs since biotherapeutic products usually consist of relatively large and complex entities that are difficult to characterize. In addition, SBPs are manufactured and controlled by processes established by the SBP manufacturer since the manufacturer of an SBP normally does not have access to all the necessary manufacturing information on the originator product. However, minor differences in the manufacturing process may affect the pharmacokinetics, pharmacodynamics, efficacy and/or safety of biotherapeutic products. It has consequently been agreed that the normal method for licensing generic medicines through bioequivalence studies alone is not scientifically appropriate for SBPs.

Decision-making regarding the licensing of SBPs should be based on scientific evidence. The onus is on the manufacturer of an SBP to provide the necessary evidence to support all aspects of an application for licensing. As in any drug development programme, development of an SBP is a stepwise approach that starts with characterization and evaluation of quality attributes of the product and is followed by nonclinical and clinical studies. Comprehensive characterization and comparison showing similarity at the quality level are the basis for possible data reduction in the nonclinical and clinical development. If differences between the
SBP and the RBP are found at any step, the underlying reasons for the differences should be investigated. Differences should always be fully explained and justified and may lead to additional data (e.g. on safety) being required.

In addition to quality data, nonclinical and clinical data are required for any SBP, generated with the product itself. The amount of such data that is considered necessary will depend on the product or class of products, on the extent of characterization possible using state-of-the-art analytical methods, on observed or potential differences between the SBP and the RBP, and on clinical experience with the product class (e.g. safety/immunogenicity concerns in a specific indication). A case-by-case approach is clearly needed for each class of products.

An SBP is intended to be similar to a licensed biotherapeutic product for which substantial evidence exists of safety and efficacy. Authorization of the SBP on the basis of reduced nonclinical and clinical data depends on proof of its similarity to an appropriate RBP through the comparability exercise. Manufacturers should demonstrate both a full understanding of their product and consistent and robust manufacture, and should submit a full quality dossier that includes a complete characterization of the product. Comparison of the SBP and the RBP with respect to quality represents an additional element to the “traditional” full quality dossier. A reduction in data requirements is therefore possible only for the nonclinical and/or clinical parts of the development programme. The dosage form and route of administration of the SBP should be the same as for the RBP.

Studies must be comparative in nature and must employ analytical methods that are capable of detecting potential differences between the SBP and the RBP. The main clinical studies should use the final formulation of the SBP, i.e. derived from the final process material, otherwise, additional evidence will be required to demonstrate that the SBP to be marketed is comparable to that used in the main clinical studies.

If similarity between the SBP and the RBP has been convincingly demonstrated, and if the manufacturer can provide scientific justification for such extrapolation, the SBP may be approved for use in other clinical indications for which the RBP is used but which have not directly been tested in clinical trials (see section 10.7). Any significant differences between the SBP and the chosen RBP detected during the comparability exercise would indicate that the products are not similar and that more extensive nonclinical and clinical data may be required to support the application for licensing.

5.1 Comparability exercise

The comparability exercise is designed to show that the SBP has quality attributes that are highly similar to those of the RBP. To provide an integrated and comprehensive set of comparative data, however, it must also include the nonclinical and clinical studies. At the level of quality, the comparability data can
be considered as additional data, over and above what is normally required for an originator product developed as a new and independent product; this is the basis for reducing the requirements for nonclinical and clinical data.

It is important that a distinction be made between the usual quality data requirements and those presented as part of the comparability exercises. It may be useful to present these as a separate section in the quality module.

### 6. Key principles for the licensing of SBPs

- The development of an SBP involves stepwise comparability exercise(s) starting with comparison of the quality characteristics of the SBP and the RBP. Demonstration of similarity of an SBP to an RBP in terms of quality is a prerequisite for reducing the nonclinical and clinical data set required for licensure. After each step of the comparability exercise, the decision to proceed further with the development of the SBP should be evaluated.

- The licensing of a product as an SBP depends on its demonstrated similarity to a suitable RBP in quality, nonclinical and clinical parameters. The decision to license the product should be based on evaluation of the whole data package for each of these parameters.

- If relevant differences between the SBP and the RBP are found in the quality, nonclinical or clinical studies, the product is unlikely to qualify as an SBP, and a more extensive nonclinical and clinical data set will probably be required to support its application for licensure. Such a product should not qualify as an SBP as defined in these guidelines.

- If comparability exercises and/or studies with the RBP are not performed throughout the development process as outlined in this document, the final product should not be referred to as an SBP.

- SBPs are not “generic medicines” and many characteristics associated with the authorization process generally do not apply.

- Like other biotherapeutic products, SBPs require effective regulatory oversight for the management of the potential risks they pose and in order to maximize their benefits.

### 7. Reference biotherapeutic products (RBPs)

Comprehensive information on the RBP provides the basis for establishing the safety, quality and effectiveness profile to which the SBP is compared. The RBP
also provides the basis for dose selection and route of administration, and is used in the comparability studies required to support the licensing application. The demonstration of an acceptable level of similarity between the SBP and RBP provides the rationale for a reduced nonclinical and clinical data set to support the application for market authorization for the SBP. The RBP is thus central to the licensing of an SBP.

To support licensure of the SBP, similarity of the SBP to the RBP should be demonstrated through head-to-head comparisons with the RBP. The same RBP should be used throughout the entire comparability exercise.

The choice of an RBP is critically important for the evaluation of the SBP. The rationale for the choice of RBP should be provided by the manufacturer of the SBP in the submission to the NRA. Traditionally, NRAs have required the use of a nationally licensed reference product for licensing of generic medicines. This practice may not be feasible for countries that lack nationally licensed RBPs, and NRAs may need to consider establishing additional criteria to guide the acceptability of using an RBP licensed or resourced in other countries. The use of reference products with proven efficacy and safety in a given population will be one of the factors to consider; another factor may be market experience in addition to the duration and volume of marketed use.

### 7.1 Considerations for choice of RBP

Since the choice of RBP is essential to the development of an SBP, the following should be considered.

- The RBP should have been marketed for a suitable duration and have a volume of marketed use such that the demonstration of similarity to it brings into relevance a substantial body of acceptable data regarding the safety and efficacy.
- The manufacturer must demonstrate that the chosen RBP is suitable to support the application for marketing authorization of an SBP.
- The RBP should have been licensed on the basis of full quality, safety, and efficacy data. An SBP should therefore not be chosen as an RBP.
- The same RBP should be used throughout the development of the SBP (i.e. throughout the comparative quality, nonclinical, and clinical studies).
- The drug substance of the RBP and the SBP must be shown to be similar.
- The dosage form and route of administration of the SBP should be the same as that of the RBP.
- The following factors should be considered in the choice of an RBP that is marketed in another jurisdiction.
- The RBP should be licensed and widely marketed in another jurisdiction that has a well-established regulatory framework and principles, as well as considerable experience of evaluation of biotherapeutic products and post-marketing surveillance activities.
- The acceptance of an RBP for evaluation of an SBP in a particular country does not imply that the NRA of that country has approved the RBP for use.

8. Quality

The quality comparison showing molecular similarity between the SBP and the RBP provides the essential rationale for predicting that the clinical safety and efficacy profile of the RBP should also apply to the SBP, meaning that the extent of the nonclinical and clinical data required for the SBP can be reduced. Ideally, development of an SBP involves thorough characterization of a number of representative lots of the RBP and then engineering a manufacturing process that will yield a product highly similar to the RBP in all clinically relevant quality attributes, i.e. product attributes that may impact clinical performance. An SBP is generally derived from a separate and independent master cell bank using independent manufacturing processes and control. These should be selected and designed to meet the required comparability criteria. A full quality dossier for both drug substance and drug product is always required and must comply with the standards required by NRAs for originator products.

Increased knowledge of the relationship between biochemical, physicochemical and biological properties of the product and clinical outcomes will facilitate development of an SBP. Because of the heterogeneous nature of proteins (especially those with extensive post-translational modifications, such as glycoproteins), the limitations of some analytical techniques, and the generally unpredictable nature of the clinical consequences of minor differences in protein structural/physicochemical properties, the evaluation of comparability will have to be carried out independently for each product. For example, oxidation of certain methionine residues in one protein may have no impact on clinical activity whereas in another protein it may significantly reduce the intrinsic biological activity or increase immunogenicity. Thus, differences in the levels of methionine oxidation in the RBP and SBP would need to be evaluated and, if present, their clinical relevance would be evaluated and discussed.

To evaluate comparability, the manufacturer should carry out a comprehensive physicochemical and biological characterization of the SBP in head-to-head comparisons with the RBP. All aspects of product quality and heterogeneity should be assessed (see section 8.2).
A high degree of similarity between the SBP and the RBP is the basis for reducing the nonclinical and clinical requirements for licensing. However, some differences are likely to be found, for example as a result of differences in impurities or excipients. Such differences should be assessed for their potential impact on clinical safety and efficacy of the SBP and justification (for example, own study results or published data) for allowing such differences should be provided. Differences of unknown clinical relevance, particularly regarding safety, may have to be addressed in additional studies pre- or post-marketing. Differences in quality attributes known to have potential impact on clinical activity will influence the decision on whether to name such a product as an SBP. For example, if differences are found in glycosylation patterns that alter the biodistribution of the product and thereby change the dosing scheme, this product cannot be considered an SBP. Other differences between the SBP and RBP may be acceptable and would not trigger the need for extra nonclinical and/or clinical evaluation. For example, a therapeutic protein that has lower levels of protein aggregates would, in most cases, be predicted to have a better safety profile than the RBP and would not need added clinical evaluation. In the same way, if heterogeneity in the terminal amino acids of the RBP is known to exist (and is adequately documented) but does not affect the bioactivity, distribution or immunogenicity of the RBP or similar products in its class, there may be no need for added clinical safety or efficacy studies based upon this heterogeneity of the RBP and SBP.

Due to the unavailability of drug substance for the RBP, the SBP manufacturer will usually be using a commercial drug product for the comparability exercise. The commercial drug product will, by definition, be in the final dosage form, containing the drug substance(s) formulated with excipients. It should be verified that these excipients do not interfere with analytical methods and thus have no impact on test results. If the drug substance in the RBP needs to be purified from a formulated reference drug product in order to be suitable for characterization, studies must be carried out to demonstrate that product heterogeneity and relevant attributes of the active moiety are not affected by the isolation process. The approach used for isolating the SBP and comparing it with the RBP should be justified and demonstrated, with data, to be appropriate for the intended purpose. Where possible, the product should be tested with and without manipulation.

8.1 Manufacturing process
Manufacture of an SBP should be based on a comprehensively designed production process, taking all relevant guidelines into account. The manufacturer must demonstrate the consistency and robustness of the manufacturing process by implementing good manufacturing practices (5), modern quality control and assurance procedures, in-process controls, and process validation. The
manufacturing process should meet the same standards as required by the NRA for originator products. It should be optimized to minimize differences between the SBP and RBP in order to (a) maximize the reduction in clinical testing requirements for the SBP based upon the clinical history of the RBP, and (b) minimize any predictable impact on the clinical safety and efficacy of the product. Some differences between the SBP and RBP are expected and may be acceptable, provided that appropriate justification of the lack of impact on clinical performance can be given.

It is understood that a manufacturer developing an SBP will not have access to confidential details of the RBP manufacturing process; thus, unless there is a contractual arrangement with the manufacturer of the RBP, the process will differ from the licensed process for the RBP. The manufacturing process for an SBP should employ state-of-the-art science and technology to achieve a high-quality product that is as similar as possible to the RBP. This will involve extensive evaluation of the RBP before the manufacturing process for the SBP is developed. The SBP manufacturer should assemble all available knowledge of the RBP regarding the type of host cell, the formulation and the container closure system used for marketing the RBP. If applicable, the SBP manufacturer should then determine the potential impact of changing any one of these elements on product quality, safety and efficacy based on available evidence from information in the public domain and experience with use of the RBP. The SBP manufacturer is encouraged to apply this knowledge to the design of the manufacturing process. The rationale for accepting these differences needs to be justified by sound science and by clinical experience with either the SBP or the RBP.

As a general rule, the product should be expressed and produced in the same host cell type as the RBP (e.g. *Escherichia coli*, Chinese hamster ovary cells, etc.) in order to minimize the potential for important changes in critical quality attributes of the protein and to avoid introduction of certain types of process-related impurities (e.g. host cell proteins, endotoxins, or yeast mannans) that could affect clinical outcomes and immunogenicity. The host cell type for manufacture of the SBP should be changed only if the manufacturer can demonstrate convincingly that the structure of the molecule is not affected or that the clinical profile of the product will not change. For example, somatropin produced in yeast cells appears to have similar characteristics to somatropin expressed in *E. coli*. In most cases, however, the use of a different host cell type will not be feasible for glycoproteins because glycosylation patterns vary significantly between different host cell types.

A complete description and data package should be provided that delineates the manufacturing process, starting with development of expression vectors and cell banks, cell culture/fermentation, harvest, purification and modification reactions, filling into bulk or final containers, and storage. The development studies conducted to establish and validate the dosage form, formulation, container closure system (including integrity to prevent microbial
Annex 2

contamination) and usage instructions should be also documented (see relevant guidelines, such as those issued by the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH)).

8.2 Characterization

Thorough characterization of both RBP and SBP should be carried out using appropriate, state-of-the-art biochemical, biophysical and biological analytical techniques. For the active ingredient(s) (i.e. the desired product), details should be provided on primary and higher-order structure, post-translational modifications (including, but not limited to, glycoforms), biological activity, purity, impurities, product-related (active) substances (variants), and immunochemical properties, where relevant.

When conducting a comparability exercise, head-to-head characterization studies are required to compare the SBP and the RBP. The primary structure of the SBP and the RBP should be identical.

If differences between the SBP and the RBP are found, their potential impact on safety and efficacy of the SBP should be evaluated. The predefined limits need to be considered in advance. Assessment of the results should include investigation of the differences found between the SBP and the RBP. This determination will be based upon knowledge of the relationship between product quality attributes and clinical activity of the RBP and related products, the clinical history of the RBP, and lot-to-lot differences for commercial lots of the RBP. For example, quality attributes such as composition and profile of glycosylation, biological activity that is known to be related to clinical activity, and receptor binding activity should be justified.

Knowledge of the analytical limitations of each technique used to characterize the product (e.g. limits of sensitivity, resolving power) should be applied when determining similarity. Representative raw data should be provided for all complex analytical methods (e.g. high-quality reproductions of gel and chromatograms) in addition to tabular data summarizing the complete data set and showing the results of all release and characterization analyses carried out on the SBP and the RBP.

The criteria outlined in the following sections should be considered when conducting the comparability exercise.

8.2.1 Physicochemical properties

The physicochemical characterization should include determination of primary and higher-order structure (secondary/tertiary/quaternary) using appropriate analytical methods (e.g. mass spectrometry or nuclear magnetic resonance) and other biophysical properties. An inherent degree of structural heterogeneity occurs
in proteins as a result of the biosynthesis process, such that the RBP and the SBP are likely to contain a mixture of post-translationally modified forms. Appropriate efforts should be made to investigate, identify and quantify these forms.

8.2.2 Biological activity

Biological activity is the specific ability or capacity of the product to achieve a defined biological effect. It serves multiple purposes in the assessment of product quality and is required for characterization and for batch analysis. Ideally, the biological assay will reflect the understood mechanism of action of the protein and will thus serve as a link to clinical activity. A biological assay is a quality measure of the “function” of the protein product and can be used to determine whether a product variant has the appropriate level of activity (i.e. a product-related substance) or is inactive (and is therefore defined as an impurity). The biological assay also complements the physicochemical analyses by confirming the correct higher-order structure of the molecule. Thus, the use of relevant biological assay(s) with appropriate precision and accuracy provides an important means of confirming that there is no significant functional difference between the SBP and the RBP.

For a product with multiple biological activities, manufacturers should perform, as part of product characterization, a set of relevant functional assays designed to evaluate the range of activities of the product. For example, certain proteins possess multiple functional domains that express enzymatic and receptor-binding activities. In such situations, manufacturers should evaluate and compare all relevant functional activities of the SBP and the RBP.

Potency is the quantitative measure of the biological activity. A relevant, validated potency assay should be part of the specification for a drug substance and/or drug product. The results of the potency assay should be provided and expressed in units of activity. Where possible (e.g. for in vitro biochemical assays, such as enzyme assays or binding assays), the results may be expressed as specific activities (e.g. units/mg protein). Assays should be calibrated against an international or national standard or reference reagent, when available and appropriate. WHO provides International Standards and Reference Reagents, which serve as reference sources of defined biological activity expressed in an international unit (IU) or unit (U). International Standards and Reference Reagents are intended for calibration of national reference standards (http://www.who.int/biologicals/reference_preparations/en/). International or national standards and Reference Reagents should therefore be used to determine the potency and to express results in IU or U. They are not intended for use as RBPs during the comparability exercise.

Biological assays can be used for purposes other than determination of potency. For example, a relevant biological assay is essential for determining
whether antibodies that develop in response to the product have neutralizing activity that affects the biological activity of the product and/or endogenous counterparts, if present (see section 10.6).

8.2.3 Immunochemical properties

When immunochemical properties are part of the characterization (e.g. for antibodies or antibody-based products), the manufacturer should confirm that the SBP is comparable to the RBP in terms of specificity, affinity, binding kinetics, and Fc functional activity, where relevant.

8.2.4 Impurities

Because access to all necessary information on the manufacturing process as well as on the drug substance of the originator product is limited, it is recognized that evaluation of the similarity of the impurity profiles of the SBP and the RBP will be generally difficult. Nevertheless, process- and product-related impurities should be identified, quantified by state-of-the-art technology and compared between the SBP and RBP. Some differences may be expected because the proteins are produced by different manufacturing processes. If significant differences in the impurity profiles of the SBP and the RBP are observed, their potential impact on efficacy and safety, including immunogenicity, should be evaluated. It is critical to have suitable assays for process-related impurities, specific to the cell line used for production.

8.3 Specifications

Specifications are employed to verify the routine quality of the drug substance and drug product rather than to fully characterize them. Specifications for an SBP, as for any biotherapeutic product, should be set as described in established guidelines and monographs, where these exist. It should be noted that pharmacopoeial monographs may provide only a minimum set of requirements for a particular product, and additional test parameters may be required. Reference to analytical methods used and acceptance limits for each test parameter of the SBP should be provided and justified. All analytical methods referenced in the specification should be validated; the corresponding validation should be documented.

Specifications for an SBP will not be the same as for the RBP since the manufacturing processes will be different and different analytical procedures and laboratories will be used for the assays. Nonetheless, the specifications should capture and control important known product quality attributes for the RBP (e.g. correct identity; purity, potency; molecular heterogeneity in terms of size, charge, and hydrophobicity, if relevant; degree of sialylation; number of individual polypeptide chains; glycosylation of a functional domain;
aggregate levels; impurities such as host cell protein and DNA). The setting of specifications should be based upon the manufacturer’s experience with the SBP (e.g. manufacturing history; assay capability; safety and efficacy profile of the product) and the experimental results obtained by testing and comparing the SBP and RBP. Sufficient lots of SBP should be employed in setting specifications. The manufacturer should demonstrate, whenever possible, that the limits set for a given specification are not significantly wider than the range of variability of the RBP over the shelf-life of the product, unless justified.

8.4 Analytical techniques

Although the power of analytical methods for characterization of proteins has increased dramatically over the past few decades, there are still obstacles to complete characterization of complex biotherapeutic products. A battery of state-of-the-art analyses is needed to determine structure, function, purity and heterogeneity of the products. The methods used should separate and analyse different variants of the product based upon different underlying chemical, physical and biological properties of protein molecules. For example, polyacrylamide gel electrophoresis (PAGE), ion exchange chromatography, isoelectric focusing, and capillary electrophoresis all separate proteins based upon charge, but they do so under different conditions and on the basis of different physicochemical properties. As a result, one method may detect variants that another method does not. The goal of the comparability investigation is to be as comprehensive as possible in order to minimize the possibility of undetected differences between the RBP and the SBP that may affect clinical activity. The analytical limitations of each technique (e.g. limits of sensitivity or resolving power) should be considered when determining the similarity between an SBP and an RBP.

The measurement of quality attributes in characterization studies (as opposed to in the specifications) does not necessarily require the use of validated assays, but the assays should be scientifically sound and qualified; that is, they should provide results that are meaningful and reliable. The methods used to measure quality attributes for lot release should be validated in accordance with relevant guidelines, as appropriate. A complete description of the analytical techniques employed for release and characterization of the product should be provided in the licence application.

8.5 Stability

The stability studies should comply with relevant guidance as recommended by the NRA. Studies should be carried out to show which release and characterization methods are stability-indicating for the product. Generally, stability studies should be summarized in an appropriate format, such as tables, and they should
include results from accelerated degradation studies and studies under various stress conditions (e.g. temperature, light, humidity and mechanical agitation).

Accelerated stability studies are an important element of the determination of similarity between an SBP and an RBP because they can reveal otherwise hidden properties of a product that warrant additional evaluation. They are also important for identifying the degradation pathways of a protein product. The results obtained from accelerated stability studies may show that additional controls should be used in the manufacturing process and during shipping and storage in order to ensure the integrity of the product. Head-to-head accelerated stability studies comparing the SBP with the RBP will be of value in determining the similarity of the products by showing a comparable degradation profile. Currently, however, stress testing carried out in a comparative manner does not provide an added value. Representative raw data showing the degradation profiles for the product should be provided in the licence application.

The stability data should support the conclusions regarding the recommended storage and shipping conditions and the shelf-life/storage period for the drug substance, drug product, and process intermediates that may be stored for significant periods of time. Stability studies on drug substance should be carried out using containers and conditions that are representative of the actual storage containers and conditions. Stability studies on drug product should be carried out in the intended drug product container-closure system. Real-time/real-temperature stability studies will determine the storage conditions and expiry dating for the product, which may or may not be the same as for the RBP.

9. Nonclinical evaluation

The nonclinical part of the Guidelines addresses the pharmacotoxicological assessment of the SBP. Establishing the safety and efficacy of an SBP usually requires the generation of some nonclinical data for the SBP.

9.1 General considerations

Demonstrating a high degree of molecular similarity between the SBP and the RBP should significantly reduce the need for nonclinical studies, since the RBP will already have a significant clinical history. Unless otherwise justified, nonclinical studies should be conducted with the final formulation of the SBP intended for clinical use.

The design of an appropriate nonclinical study programme requires a clear understanding of the product characteristics. Results from the physicochemical and biological characterization studies should be reviewed from the point of view of potential impact on efficacy and safety. In the development of an SBP, some existing guidelines (for example, ICH S6, Preclinical safety evaluation of
biotechnology-derived pharmaceuticals (6)) may be relevant and should therefore be taken into account.

SBPs often require unique approaches to assessing their safety in nonclinical studies. Problems in the nonclinical evaluation of SBPs containing biotechnology-derived recombinant proteins as drug substance are often related to the fact that these products:

- may show species-specific pharmacodynamic activity such that it is sometimes difficult to identify a relevant species for pharmacodynamic and toxicological evaluation; and/or
- will, as “foreign proteins”, usually elicit an antibody response in long-term animal studies, and the formation of antibody complexes with the drug substance may make it difficult to interpret the results of subchronic or chronic repeat-dose studies.

9.2 Special considerations

Nonclinical evaluation of a new biotherapeutic normally encompasses a broad spectrum of pharmacodynamic, pharmacokinetic and toxicological studies (6). The amount of additional nonclinical data required to establish the safety and efficacy of an SBP is considered to be highly dependent on the product and on factors related to substance class. Factors that often elicit the need for additional nonclinical studies include, but are not restricted to, the following:

- Quality-related factors:
  - significant differences in the cell expression system compared with the RBP;
  - significant differences in purification methods used;
  - the presence of a complex mixture of less well-characterized product- and/or process-related impurities.

- Factors related to pharmacotoxicological properties of the drug substance:
  - mechanism(s) of drug action are unknown or poorly understood;
  - the drug substance is associated with significant toxicity and/or has a narrow therapeutic index;
  - limited clinical experience with the RBP.

Depending on these factors, the spectrum of studies required to establish the safety and efficacy of the SBP may vary considerably and should be defined on a case-by-case basis. For example, in the case of a highly complex...
drug substance that is difficult to characterize by analytical techniques and that possesses a narrow therapeutic index, the nonclinical development programme may encompass a significant portion of the spectrum of studies described in relevant guidelines such as ICH S6 (6). On the other hand, for products for which the drug substance and the impurity profile are well characterized by analytical means, which possess a wide therapeutic index and for which extensive clinical experience is available, the nonclinical development programme will probably be more limited. However, a head-to-head repeat-dose toxicity study should usually constitute a minimum requirement for nonclinical evaluation of an SBP. The nonclinical studies constitute a part of the overall comparability exercise. They should therefore be comparative in nature and designed to detect differences in response between the SBP and the RBP and not just the response to the SBP alone. Any deviation from this approach should be appropriately justified.

9.2.1  **In vitro studies**

Assays such as receptor-binding studies or cell-based assays (e.g. cell-proliferation or cytotoxicity assays) should normally be undertaken to establish comparability of the biological/pharmacodynamic activity of the SBP and the RBP. Such data are usually already available from the biological assays described in the quality part of the dossier (see section 8.2.2). Reference to these studies can be made in the nonclinical part of the dossier.

9.2.2  **In vivo studies**

Animal studies should be designed to maximize the information obtained. They should be comparative in nature (see above), should be performed in a species known to be relevant (i.e. a species in which the RBP has been shown to possess pharmacodynamic and/or toxicological activity), and should employ state-of-the-art technology. Where the model allows, consideration should be given to monitoring a number of end-points such as:

- Biological/pharmacodynamic activity relevant to the clinical application. These data should usually be available from biological assays described in the quality part of the dossier (see section 8.2.2) and reference to these studies can be made in the nonclinical part of the dossier. If feasible, biological activity may be evaluated as part of the nonclinical repeat-dose toxicity study (described below). In vivo evaluation of biological/pharmacodynamic activity may be unnecessary if in vitro assays are available that have been validated as reliably reflecting the clinically relevant pharmacodynamic activity of the RBP.
Nonclinical toxicity as determined in at least one repeat-dose toxicity study carried out in a relevant species and including toxicokinetic measurements. Toxicokinetic measurements should include determination and characterization of antibody responses, including anti-product antibody titres, cross-reactivity with homologous endogenous proteins, and product-neutralizing capacity. The studies should be of sufficient duration to allow detection of potential differences in toxicity and antibody responses between the SBP and the RBP.

Besides being a part of the overall comparability exercise, the comparative repeat-dose toxicity study is considered to provide reassurance that no “unexpected” toxicity will occur during clinical use of the SBP. If performed with the final formulation intended for clinical use, the repeat-dose toxicity study will, in principle, allow for detection of potential toxicity associated both with the drug substance and with product- and process-related impurities.

Although the predictive value of animal models for immunogenicity in humans is considered low, antibody measurements, if applicable, should be included in the repeat-dose toxicity study to aid in the interpretation of the toxicokinetic data and in assessing, as part of the overall comparability exercise, whether important differences in structure or immunogenic impurities exist between the SBP and the RBP (the immunological response may be sensitive to differences not detected by laboratory analytical procedures).

Depending on the route of administration, local tolerance may need to be evaluated. If feasible, this evaluation may be performed as part of the described repeat-dose toxicity study.

On the basis of the demonstration of similarity between the SBP and RBP by the additional comparability exercise performed as part of the quality evaluation, other routine toxicological studies – such as safety pharmacology, reproductive toxicology, genotoxicity and carcinogenicity studies – are not generally requirements for the nonclinical testing of an SBP, unless triggered by results of the repeat-dose toxicity study or the local tolerance study and/or by other known toxicological properties of the RBP (e.g. known adverse effects of the RBP on reproductive function).

**10. Clinical evaluation**

The main/pivotal clinical data should be generated using the product derived from the final manufacturing process, which reflects the product for which marketing authorization is sought. Any deviation from this recommendation needs to be justified and additional data may be required, such as from pharmacokinetic...
bridging studies comparing the pharmacokinetic profiles of the products from the previous and final formulations. For changes in the manufacturing process, ICH Q5E should be followed (7).

Clinical studies should be designed to demonstrate comparable safety and efficacy of the SBP and the RBP and therefore need to employ testing strategies that are sensitive enough to detect any relevant differences between the products.

The clinical comparability exercise is a stepwise procedure that should begin with pharmacokinetic and pharmacodynamic studies and continue with the pivotal clinical trials. If relevant differences between the SBP and the RBP are detected at any stage, the reasons need to be explored and justified. If this is not possible, the new product may not qualify as an SBP and a full licensing (stand-alone) application should be considered.

10.1 Pharmacokinetic studies

The pharmacokinetic profile is an essential part of the basic description of a medicinal product and should always be investigated. Pharmacokinetic studies should generally be performed for the routes of administration applied for and using doses within the therapeutic dose range recommended for the RBP.

Pharmacokinetic studies must be comparative in nature and should be designed to enable the detection of potential differences between the SBP and the chosen RBP. This is usually best achieved by performing single-dose, cross-over pharmacokinetic studies in a homogenous study population and by using a dose at which the sensitivity to detect differences is greatest. For example, for a medicinal product with saturable absorption (saturation kinetics), the lowest therapeutic dose would be most appropriate, provided that the assay used can measure the resulting drug plasma levels with sufficient accuracy and precision. To reduce any variability that is unrelated to differences between products, pharmacokinetic studies could be performed in healthy volunteers (if considered ethical and scientifically justified). If the drug substance under investigation is known to have adverse effects and the pharmacological effects or risks are considered unacceptable for healthy volunteers, it may be necessary to perform the pharmacokinetic studies in the proposed patient population.

In general, single-dose pharmacokinetic studies will suffice. However, in cases of dose- or time-dependent pharmacokinetics, resulting in markedly higher concentrations at steady-state than would be expected from single-dose data, a potential difference in the extent of absorption of the SBP and RBP may be greater at steady state than after single-dose administration. In such cases, it may be advisable for the manufacturer to perform an additional comparative multiple-dose study, to ensure that pharmacokinetic profiles are also similar at steady state, before starting the confirmatory clinical trial(s). In steady-state pharmacokinetic studies, the administration scheme should preferably use the highest dosage customarily recommended for the RBP.
The choice of single-dose studies, steady-state studies or repeated determination of pharmacokinetic parameters, and of the study population should be justified by the manufacturer. The cross-over design eliminates inter-subject variability and therefore, compared with the parallel design, reduces the sample size necessary to show equivalent pharmacokinetic profiles of the SBP and RBP. The treatment phases should be separated by an adequate wash-out phase to avoid carry-over effects. The cross-over design may not be appropriate for biological medicinal products with a long half-life or for proteins that are likely to provoke the formation of anti-product antibodies. In parallel designs, care should be taken to avoid relevant imbalances in all prognostic variables between treatment groups that may affect the pharmacokinetics of the drug substance (e.g. ethnic origin, smoking status, and metabolizer status of the study population).

Pharmacokinetic comparison of the SBP and the RBP should include not only absorption/bioavailability but also elimination characteristics, i.e. clearance and/or elimination half-life, which may differ between the SBP and the RBP.

Acceptance criteria for the demonstration of pharmacokinetic similarity between the SBP and the RBP should be predefined and appropriately justified. It should be noted that the criteria used in standard clinical pharmacokinetic comparability studies (bioequivalence studies) were developed for chemically-derived, orally administered products and may not necessarily be applicable for biotherapeutic products. The lack of established acceptance criteria designed for biologicals means that the traditional 80–125% equivalence range is often used. However, if the 90% confidence intervals of the ratio of the population geometric means (test/reference) for the main parameters under consideration (usually rate and extent of absorption) fall outside this traditional range, the SBP may still be considered similar to the RBP provided that there is sufficient evidence of similarity from the quality, nonclinical, pharmacodynamic, efficacy and safety comparisons.

Other pharmacokinetic studies, such as interaction studies (with drugs likely to be used concomitantly) or studies in special populations (e.g. children, the elderly and patients with renal or hepatic insufficiency), are not usually required for an SBP.

Historically, limitations in the assay methodology for pharmacokinetic evaluation of peptide or protein products have restricted the usefulness of such studies. There should consequently be special emphasis on the analytical method selected and its ability to detect and follow the time course of the protein (the parent molecule and/or degradation products) in a complex biological matrix that contains many other proteins. The method should be optimized to provide satisfactory specificity, sensitivity and a range of quantification with adequate accuracy and precision.
In some cases, the presence of measurable concentrations of endogenous protein may substantially affect the measurement of the concentration–time profile of the administered exogenous protein. In such cases, the manufacturer should describe and justify the approach to minimize the influence of the endogenous protein on the results.

10.2 Pharmacodynamic studies

Although comparative clinical trials are usually required to demonstrate the similar efficacy and safety of the SBP and RBP, it may be advisable for the manufacturer to ensure similar pharmacodynamic profiles before proceeding to clinical trials, particularly if a difference in pharmacokinetic profiles, of unknown clinical relevance has been detected.

In many cases, pharmacodynamic parameters are investigated in the context of combined pharmacokinetic/pharmacodynamic studies. Such studies may provide useful information on the relationship between dose/exposure and effect, particularly if performed at different dose levels. In the comparative pharmacodynamic studies, pharmacodynamic effects should be investigated in a suitable population using a dose or doses within the steep part of the dose–response curve in order to maximize the chance of detecting potential differences between the SBP and the RBP. Pharmacodynamic markers should be selected on the basis of their clinical relevance.

10.3 Confirmatory pharmacokinetic/pharmacodynamic studies

Clinical trials are usually required to demonstrate similar efficacy of the SBP and the RBP. In certain cases, however, comparative pharmacokinetic/pharmacodynamic studies may be appropriate, provided that:

- the pharmacokinetic and pharmacodynamic properties of the RBP are well characterized;
- at least one pharmacodynamic marker is a marker linked to efficacy (e.g. an accepted surrogate marker for efficacy); and
- the relationship between dose/exposure, the relevant pharmacodynamic marker(s) and response/efficacy of the RBP is established.

Euglycaemic clamp studies would be an example for acceptable confirmatory pharmacokinetic/pharmacodynamic studies for comparing the efficacy of two insulins. In addition, absolute neutrophil count and CD34+ cell count are the relevant pharmacodynamic markers for the activity of granulocyte colony stimulating factor (G-CSF) and could be used in pharmacokinetic/pharmacodynamic studies in healthy volunteers to demonstrate the similar efficacy of two G-CSF-containing medicinal products.
The study population and dosage should represent a test system that is known to be sensitive to potential differences between the SBP and the RBP. In the case of insulin, for example, the study population should consist of non-obese healthy volunteers or patients with type 1 diabetes rather than insulin-resistant obese patients with type 2 diabetes. Otherwise, it will be necessary to investigate a relevant dose range to demonstrate that the test system is discriminatory (8). In addition, the acceptance ranges for demonstration of similarity in confirmatory pharmacokinetic and pharmacodynamic parameters should be predefined and appropriately justified. If appropriately designed and performed, such pharmacokinetic/pharmacodynamic studies are often more sensitive in detecting potential differences in efficacy than trials using clinical end-points.

10.4 Efficacy studies

Dose-finding studies are not required for an SBP. Demonstration of comparable potency, pharmacokinetic and pharmacodynamic profiles provide the basis for use of the RBP posology in the confirmatory clinical trial(s).

Similar efficacy of the SBP and the chosen RBP will usually have to be demonstrated in adequately powered, randomized and controlled clinical trial(s). The principles of such trials are laid down in relevant ICH guidelines (8, 9). Clinical studies should preferably be double-blind or at a minimum observer-blind. In the absence of any blinding, careful justification will be required to prove that the trial results are free from significant bias.

Potential differences between the SBP and the RBP should be investigated in a sensitive and preferably well-established clinical model. In the case of growth hormone (GH), for example, treatment-naive children with GH deficiency usually represent the most appropriate study population, as opposed to children with non GH-deficient short stature who are usually less sensitive to the effects of GH. Although adult patients with GH deficiency could also be considered a “sensitive” population, the end-point used to measure the effects of GH treatment (body composition) is less sensitive than the one used in children (longitudinal growth), making an equivalence or non-inferiority margin more difficult to define.

In principle, equivalence designs (requiring lower and upper comparability margins) are clearly preferred for comparing the efficacy and safety of the SBP and the RBP. Non-inferiority designs (requiring only one margin) may be considered if appropriately justified. While both designs can be used, their advantages and disadvantages should be well understood. The designs should be chosen with due regard to the possible advantages and disadvantages of each (see “Advantages and disadvantages of equivalence/non-inferiority designs for SBPs” below). For statistical considerations see section “Statistical considerations for the design and analysis of equivalence/non-inferiority trials for SBPs” below.

Equivalence/non-inferiority margins must be prespecified and justified on the basis of clinical relevance; that is, the selected margin should represent the
largest difference in efficacy that would not matter in clinical practice. Treatment differences within this margin would thus, by definition, be acceptable because they have no clinical relevance.

Similar efficacy implies that similar treatment effects can be achieved when using the same dosage(s); in the head-to-head comparative trial(s), the same dosage(s) of SBP and RBP should be used. In cases where the medicinal product is titrated according to treatment response (e.g. epoetin or insulin) rather than being given at a fixed dosage (e.g. somatropin in GH-deficient children), equivalence/non-inferiority should be demonstrated with regard not only to treatment response but also to dosage. This is best achieved by defining co-primary end-points that also include dosage.

Generally, equivalence trials are clearly preferable to ensure that the SBP is not clinically less or more effective than the RBP when used at the same dosage(s). For medicinal products with a wide safety margin, non-inferiority trials may also be acceptable. However, it should be considered that non-inferior efficacy, by definition, does not exclude the possibility of superior efficacy of the SBP compared with the RBP; this, if clinically relevant, would contradict the principle of similarity.

Before starting the confirmatory clinical trial, all comparative data generated up to this point should therefore be carefully reviewed and analysed to ascertain similarity of the SBP and the RBP. The confirmatory trial marks the last step of the comparability exercise and prior demonstration of similar physicochemical characteristics, potency and pharmacokinetic/pharmacodynamic profiles make superior efficacy of the SBP compared with the RBP highly unlikely. However, in the rare event that, after completion of the study, the results indeed indicate statistically superior efficacy, any clinical relevance of this superiority should be excluded: it could be associated with increased adverse events if the SBP is prescribed at the same dosage as the RBP. In the case of an equivalence trial, clinically meaningful differences – including superior efficacy – between the SBP and the RBP are excluded if the 95% confidence interval of the treatment difference is fully contained within the prespecified two-sided (upper and lower) comparability margins. In the case of a non-inferiority trial, a post-hoc justification of superior efficacy, if observed, having no clinical relevance may be more difficult.

Whatever the predefined study design, the real results obtained from the clinical trial(s) will determine whether the SBP and the RBP can be considered to be clinically similar. If clinically relevant differences are found, the new product should not be considered to be similar to the RBP and should be developed as a stand-alone product.

Whereas several examples exist for licensing of SBPs based on equivalence trials (e.g. recombinant human GH, epoetin and G-CSF in the European Union), experience with non-inferiority trials for this purpose is limited and
based principally on theoretical considerations. An additional advantage of demonstrating equivalent efficacy (rather than non-inferior efficacy) is that this would provide a stronger rationale for the possibility of extrapolation of efficacy data to other indications of the RBP, particularly if these include different dosages from that (or those) tested in the clinical trial (see section 10.7).

10.4.1 Advantages and disadvantages of equivalence/non-inferiority designs for SBPs

An equivalence trial is designed to confirm the absence of a clinically meaningful difference between the SBP and the RBP. This is the most suitable design for confirming that the SBP is equivalent to the RBP; this is in line with the principle of similarity, since a non-inferiority trial does not exclude the possibility that the SBP is shown to be statistically and clinically superior to the RBP (which contradicts the principle of similarity). Table A2.1 below highlights the advantages and disadvantages of each design.

10.4.2 Statistical considerations for the design and analysis of equivalence/non-inferiority trials for SBPs

As indicated above, equivalence or non-inferiority studies may be acceptable for the comparison of efficacy and safety of the SBP and the RBP. The choice of clinical trial design will depend on the product in question, its intended use, disease prevalence and the target population. The specific design selected for a particular study should be clearly stated in the trial protocol and justified. Complex, and often very subtle, statistical issues are involved in the design, analysis and interpretation of equivalence and non-inferiority trials. This section is intended to emphasize the importance of the points that need to be considered in designing and analysing equivalence and non-inferiority trials; it does not provide a comprehensive overview of all statistical considerations. In particular, a good understanding of statistical confidence intervals and their application to equivalence and non-inferiority clinical trials is essential.

Irrespective of the trial design selected, a comparability margin should be specified during trial design and clearly documented in the study protocol. For an equivalence trial, both the lower and upper equivalence margins are required, while only one margin is required for a non-inferiority trial. The selection of the margin should be given careful consideration and should be justified both statistically and clinically. Adequate evidence of the effect size of the RBP should be provided to support the proposed margin. The magnitude and variability of the effect size of the RBP derived from historical trials should also be taken into consideration in determining the comparability margin in terms both of the end-point chosen and of the population to be studied. There must be reasonable assurance that the study is capable of showing any difference that exists between the RBP and SBP; this is referred to as “assay sensitivity”.
Table A2.1
Advantages and disadvantages of equivalence/non-inferiority designs for SBPs

<table>
<thead>
<tr>
<th>Design</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equivalence</td>
<td>Demonstration of equivalence provides a strong rationale for the possibility of extrapolation of efficacy to other indications of the RBP.</td>
<td>An equivalence trial tends to need a larger sample size to achieve the same study power as a non-inferiority trial. A finding of superiority would lead to the failure of the equivalence trial. There would be no option to show that the superiority observed is not clinically relevant. However, a stand-alone application might still be an option, subject to a requirement for additional studies.</td>
</tr>
<tr>
<td></td>
<td>Current experience for the licensing of SBPs is based on equivalence trials.</td>
<td></td>
</tr>
<tr>
<td>Non-inferiority</td>
<td>A non-inferiority trial requires a smaller sample size to achieve the same study power as an equivalence trial. A finding of superiority of the SBP compared to the RBP would not lead to failure of a non-inferiority trial, provided that it can be demonstrated that the superiority observed is not clinically relevant.</td>
<td>Post-hoc justification that a finding of statistically superior efficacy is not clinically relevant is difficult. If the superiority observed is considered clinically relevant, the SBP would not be considered to be similar to the RBP and should be developed as a stand-alone product. Demonstration that superior efficacy of the SBP, prescribed at the same dosage as the RBP, is not associated with increased adverse events would be required in all cases. Demonstration of non-inferiority does not provide a strong rationale for the possibility of extrapolation to other indications of the RBP. There is currently no experience with licensing of SBPs based on non-inferiority trials.</td>
</tr>
</tbody>
</table>

Statistical analysis for both equivalence and non-inferiority designs is generally based on the use of two-sided confidence intervals (typically at the 95% level) for the difference between treatments. For equivalence trials, equivalence is demonstrated when the entire confidence interval falls within the lower and upper equivalence margins. Non-inferiority evaluations are one-sided and statistical inference is based only on the lower or upper confidence limit, whichever is appropriate for a given study. For example, if a lower margin is defined, non-inferiority is demonstrated when the lower limit of the confidence interval is above the non-inferiority margin. Analysis of non-inferiority trials can also be based on a one-sided confidence interval at the 97.5% level.
Details of the sample size calculations should be provided in the study protocol. The basis of estimates of any quantities used in the sample size calculation should also be clearly explained, and these estimates will usually be based on results from earlier trials with the RBP or on published literature. Since the formulae for sample size calculations are slightly different between equivalence and non-inferiority trials, and the two-sided equivalence trial tends to need a larger sample size than a one-sided non-inferiority trial, sample size calculations should be based on methods specifically designed for equivalence or non-inferiority trials. In estimating the sample size for equivalence or non-inferiority trials, it is usually assumed that there is no difference between the SBP and the RBP. An equivalence trial could be underpowered if the true difference is not zero. Similarly, a non-inferiority trial could be underpowered if the SBP is actually less effective than the RBP. Determination of the appropriate sample size is dependent on various factors including: the type of primary end-point (e.g. binary, quantitative or time-to-event), the predefined comparability margin, the probability of a type I error (falsely rejecting the null hypothesis) and the probability of a type II error (erroneously failing to reject the null hypothesis). Keeping the probability of a type II error low will increase the ability of the study to show equivalence or non-inferiority of the SBP to the RBP. The expected rates of patient dropouts and withdrawals should also be taken into consideration in the determination of the sample size.

10.5 Safety

Pre-licensing safety data should be obtained in a sufficient number of patients to characterize the safety profile of the SBP. Depending on their size and duration, efficacy trials may be sufficient or may need to be extended to provide an adequate safety database. Comparison with the RBP should include type, frequency and severity of adverse events/reactions. For cases in which similar efficacy is demonstrated in confirmatory pharmacokinetic/pharmacodynamic studies but safety data relevant for the target population cannot be deduced from these studies, data on safety in the target population are still needed. For example, for two soluble insulins, the euglycaemic clamp study is considered the most sensitive method for detecting differences in efficacy. However, immunogenicity and local tolerance of subcutaneously administered SBP cannot be assessed in such a study and should therefore be evaluated in the target population.

Safety data should preferably be comparative. Comparison with an external control group is usually hampered by differences in the investigated patient population and concomitant therapy, observation period and/or reporting. Safety data obtained from the clinical trials can be expected mainly to detect frequent and short-term adverse events/reactions. Such data are usually sufficient pre-licensing, but further close monitoring of clinical safety of the SBP is usually necessary in the post-marketing phase (see section 11).
10.6 **Immunogenicity**

Immunogenicity of biotherapeutic products should always be investigated pre-authorization. Even if efficacy and safety of an SBP and RBP have been shown to be similar, immunogenicity may still be different.

The immune response to a biotherapeutic is influenced by many factors including the nature of the drug substance, product- and process-related impurities, excipients and stability of the product, route of administration, dosing regimen, and patient-, disease- and/or therapy-related factors (10).

The consequences of unwanted immunogenicity may vary considerably, from the clinically irrelevant to the serious and life-threatening. Although neutralizing antibodies directly alter the pharmacodynamic effect of a product (i.e. by directly blocking an active site of the protein), binding antibodies often affect pharmacokinetics and thereby also influence pharmacodynamics. Thus, an altered effect of the product as a consequence of anti-product antibody formation might be a composite of pharmacokinetic, pharmacodynamic and safety effects.

Immunogenicity of a biotherapeutic should always be investigated in humans since animal data are usually not predictive of the immune response in humans. The frequency and type of antibodies induced, as well as the possible clinical consequences of the immune response, should be compared for the SBP and the RBP. Comparison with an external control group is not considered appropriate because it is usually hampered by differences in the investigated patient population, observation period, sampling time points, assays employed, and interpretation of results.

Generally, the amount of immunogenicity data obtained from the comparative efficacy trial(s) (i.e. trials that are powered for their primary efficacy end-point) will allow detection of a marked increase in immunogenicity of the SBP compared with the RBP and will be sufficient pre-licensing. Where clinically meaningful or even serious antibody development has been encountered with the RBP (or the substance class) but is too rare to be captured pre-licensing (e.g. cross-reacting neutralizing anti-epoetin antibodies causing pure red cell aplasia), a specific risk management plan for the SBP may be necessary to assess this specific risk post-marketing (see section 11). In case similar efficacy is demonstrated in confirmatory pharmacokinetic/pharmacodynamic studies, immunogenicity data in the target population are still needed (see section 10.5). If the manufacturer intends to extrapolate efficacy and safety data to other approved indications of the RBP (see section 10.7), care should be taken to ensure that immunogenicity is investigated in the patient population that carries the highest risk of an immune response and immune-related adverse events.

The manufacturer will need to justify its antibody testing strategy including the selection, assessment and characterization of assays, identification of appropriate sampling time points including baseline, sample volumes and sample processing/storage as well as selection of statistical methods for analysis.
of data. Antibody assays need to be validated for their intended purpose. A screening assay of sufficient sensitivity should be used for antibody detection and a neutralization assay should be available for further characterization of antibodies, if present. Possible interference of the circulating antigen with the antibody assay(s) should be taken into account. Detected antibodies need to be further characterized and their potential clinical implications for safety, efficacy and pharmacokinetics evaluated. For example, the isotype of the antibodies should be determined if they may be predictive of safety (e.g. development of IgE antibodies correlates with the development of allergic and anaphylactic responses). If the antibody incidence is higher with the use of the SBP than with the RBP, the reason for the difference needs to be investigated. Special attention should be paid to the possibility that the immune response seriously affects the endogenous protein and its unique biological function.

The required observation period for immunogenicity testing will depend on the intended duration of therapy and the expected time of antibody development and should be justified by the manufacturer. In the case of chronic administration, one-year data will usually be appropriate pre-licensing to assess antibody incidence and possible clinical implications. This is the case, for example, for somatropin-containing products, where antibody development usually occurs within the first 6–9 months of treatment but potential effects on growth are only seen thereafter. In some cases, shorter pre-licensing observation periods may be sufficient; for insulins, for example, most susceptible patients will develop antibodies within the first 6 months of treatment and clinical consequences, if any, would usually be observed at about the same time as antibody development. If considered clinically relevant, development of antibody titres, their persistence over time, potential changes in the character of the antibody response and the possible clinical implications should be assessed pre- and post-marketing.

Since pre-licensing immunogenicity data are often limited, further characterization of the immunogenicity profile may be necessary post-marketing, particularly if rare antibody-related serious adverse events may occur that are not likely to be detected in the pre-marketing phase.

10.7 Extrapolation of efficacy and safety data to other clinical indications

If similar efficacy and safety of the SBP and RBP have been demonstrated for a particular clinical indication, extrapolation of these data to other indications of the RBP (not studied in independent clinical studies with the SBP) may be possible if all of the following conditions are fulfilled:

- A sensitive clinical test model has been used that is able to detect potential differences between the SBP and the RBP.
The clinically relevant mechanism of action and/or involved receptor(s) are the same; e.g. GH action in different conditions of short stature in children; erythropoiesis-stimulating action of epoetins in different conditions associated with anaemia or for the purpose of autologous blood donation. If the mechanism of action is different or not known, a strong scientific rationale and additional data (e.g. “pharmacodynamic fingerprint”, additional clinical data) will be needed.

Safety and immunogenicity of the SBP have been sufficiently characterized and no unique or additional safety issues are expected for the extrapolated indication(s), for which clinical data on the SBP are not being provided; e.g. immunogenicity data in immunosuppressed patients would not allow extrapolation to an indication in healthy subjects or patients with autoimmune diseases, although the reverse would be valid.

If the efficacy trial used a non-inferiority study design and demonstrated acceptable safety and efficacy of the SBP compared to the RBP, the applicant should provide convincing arguments that this finding can be applied to the extrapolated indications; e.g. results from a non-inferiority trial in an indication where a low dose is used may be difficult to extrapolate to an indication where a higher dose is used, from the standpoint of both efficacy and safety.

If these prerequisites for extrapolation of efficacy and safety data of the SBP to other indication(s) of the RBP are not fulfilled, the manufacturer will need to submit clinical data to support the desired indication(s).

If extrapolation of results from clinical studies for one indication to one or more different indications is intended, a detailed scientific discussion on the risk–benefit of such a proposal should be provided, based on the above criteria.

11. Pharmacovigilance

As for most biological medicines, data from pre-authorization clinical studies are usually too limited to identify all potential unwanted effects of an SBP. In particular, rare adverse events are unlikely to be encountered in the limited clinical trial populations being tested with the SBP. Further close monitoring of the clinical safety of an SBP in all approved indications and a continued benefit–risk assessment are therefore necessary in the post-marketing phase.

The manufacturer should submit a safety specification and pharmacovigilance plan at the time of submission of the marketing authorization application. The principles of pharmacovigilance planning can be found in
relevant guidelines such as ICH E2E (11). The safety specification should describe important identified or potential safety issues for the RBP and for the substance class and/or any that are specific for the SBP. The pharmacovigilance plan should describe the planned post-marketing activities and methods based on the safety specification (11). In some cases, risk minimization measures such as educational material for patients and/or treating physicians may enhance the safety of using the SBP.

Any specific safety monitoring imposed on the RBP or product class should be incorporated into the pharmacovigilance plan for the SBP, unless a compelling justification can be provided to show that this is not necessary. Moreover, potential additional risks identified during the review of the data obtained with the SBP should be subject to further safety monitoring (e.g. increased immunogenicity that might result from a difference in the glycosylation profile).

Post-marketing safety reports should include all information on product tolerability received by the marketing authorization holder. The safety information must be evaluated in a scientific manner and should include evaluation of the frequency and causality of adverse events.

Manufacturers should ensure that, at the time of the marketing authorization, they have in place an appropriate pharmacovigilance system, including the services of a qualified person responsible for monitoring pharmacovigilance and the necessary means for notification of adverse reactions that occur in any of the countries where the product is marketed.

After the marketing authorization is granted, it is the responsibility of the NRA to monitor closely the compliance of manufacturers with their marketing commitments, where appropriate, and particularly with their pharmacovigilance obligations (as previously described).

In addition, as for all biotherapeutics, an adequate system for ensuring specific identification of the SBPs (i.e. traceability) is essential. The NRA shall provide a legal framework for proper pharmacovigilance surveillance and ensure the ability to identify any biotherapeutic marketed in its territory that is the subject of adverse reaction reports. This implies that an adverse reaction report for any biotherapeutic should include, in addition to the International Nonproprietary Name (INN) (12), other important indicators such as proprietary (brand) name, manufacturer’s name, lot number and country of origin.

12. Prescribing information and label

The SBP should be clearly identifiable by a unique brand name. Where an INN is defined, this should also be stated; WHO policy on INN should be followed (http://www.who.int/medicines/services/inn/innquidance/en/index.html).
Provision of the lot number is essential; it is an important part of production information and critical for traceability whenever problems with a product are encountered.

The prescribing information for the SBP should be as similar as possible to that of the RBP except for product-specific aspects, such as different excipient(s). This is particularly important for posology and safety-related information, including contraindications, warnings and adverse events. However, if there are fewer indications for the SBP than for the RBP, the related text in various sections may be omitted unless it is considered important to inform doctors and patients about certain risks, e.g. as a result of potential off-label use. In such cases it should be clearly stated in the prescribing information that the SBP is not intended for use in the specific indication(s) and the reasons why. The NRA may choose to mention in the product information the SBP nature of the product, the studies that have been performed with the SBP and the specific RBP, and/or to include instructions for the prescribing physician on how to use SBP products.

13. Roles and responsibilities of national regulatory authorities

One of the responsibilities of an NRA is to set up appropriate regulatory oversight for the licensing and post-marketing surveillance of SBPs that are developed and/or authorized for use in its area of jurisdiction. The experience and expertise of the NRA in evaluating biotherapeutic products is a key prerequisite for appropriate regulatory oversight of these products. The NRA is responsible for determining a suitable regulatory framework for licensing SBPs. It may choose to use or amend existing pathways or to develop a new pathway for this purpose.

As development of biotherapeutic products is a rapidly evolving area, regular review of NRAs for their licensing, for adequacy of their regulations for providing oversight, and for the processes and policies that constitute the regulatory framework is an essential component of a well-functioning and up-to-date regulatory oversight for biotherapeutics.

An NRA may have the necessary legal basis to approve all new drugs and as such may not need to amend its regulations to authorize SBPs. However, the European Union has specifically amended its regulations to provide an abbreviated regulatory pathway for SBPs (biosimilars) (13–16). This issue is the subject of discussion in a number of other countries where SBPs are being developed. Health Canada and Japan, for example, have recently developed their guidelines for manufacturers, and national guidelines are under development in a number of other countries. The historical perspective of the United States Food and Drug Administration on the assessment of follow-on protein products has also been published (17). In most instances, NRAs will need to provide guidance...
to manufacturers on the information needed and regulatory requirements for the authorization of SBPs. Most countries will either use or amend their existing legislation and applicable regulations or will develop entirely novel frameworks for the authorization of SBPs. In some jurisdictions, regulations for licensing subsequent entry versions of biotherapeutic products are intricately linked with policies for innovation. Hence an NRA may need to coordinate with other stakeholders for consistency.

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References


Annex 3

Recommendations to assure the quality, safety and efficacy of pneumococcal conjugate vaccines


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Recommendations published by WHO are intended to be scientific and advisory. Each of the following sections constitutes guidance for national regulatory authorities and for manufacturers of biological products. If a national regulatory authority so desires, these Recommendations may be adopted as definitive national requirements, or modifications may be justified and made by the regulatory authority. It is recommended that any modifications to these Recommendations be made only on condition that they 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 intended for the additional guidance of manufacturers and national regulatory authorities that may benefit from those details.
Introduction

These Recommendations provide guidance for the production and control of pneumococcal conjugate vaccines in Part A and for their nonclinical evaluation in Part B. Part C covers the clinical development programme applicable to pneumococcal conjugate vaccines intended primarily for the prevention of invasive pneumococcal disease (IPD) and for administration to infants and toddlers. Clinical assessment of the potential of these vaccines to prevent IPD in older children and adults (including the elderly) or to prevent other types of pneumococcal infection (e.g. pneumonia and otitis media) is not considered in any detail.

General considerations

Infections caused by *Streptococcus pneumoniae* are responsible for substantial morbidity and mortality, particularly in the very young and in the elderly (1–3). Pneumococci are grouped into more than 90 serotypes on the basis of their chemically and serologically distinct capsular polysaccharides. Certain serotypes are much more likely than others to be associated with clinically apparent infections, to cause severe invasive infections and to acquire resistance to one or more classes of antibacterial agents (4).

The capsular polysaccharides of 23 serotypes are included in licensed non-conjugated polysaccharide vaccines produced by various manufacturers. Non-conjugated pneumococcal polysaccharide vaccines elicit T-cell-independent immune responses; as a result, they do not elicit protective immune responses in children under the age of about 2 years, nor do they induce immune memory. Moreover, they have little or no impact on nasopharyngeal carriage (5). However, they are widely recommended for use in the elderly and in subjects from the age of approximately 2 years with underlying medical conditions that put them at high risk of developing IPD (6).

The development of pneumococcal conjugate vaccines, in which each of the selected bacterial capsular polysaccharides is coupled with a protein carrier molecule, has been a major advance in the prevention of IPD (7–10). In contrast to the 23-valent non-conjugated vaccines, conjugated vaccines induce T-cell-dependent immunity. They are consequently immunogenic in infants under 2 years of age and they elicit immune memory. Since 2006, WHO has recommended that all countries should incorporate pneumococcal conjugate vaccines in routine immunization schedules for children aged less than 2 years and has prioritized their introduction in countries with high child mortality rates and/or high rates of human immunodeficiency virus (HIV) infection (6).

A 7-valent pneumococcal conjugate vaccine (7vPnC) that employs CRM197 as the carrier protein for all seven serotypes was the first to be
developed. It was first licensed in the USA in 2000 and has subsequently become available in some 90 countries worldwide. Pneumococcal conjugate vaccines that contain three (11) or six serotypes, in addition to those in the 7vPnC vaccine, have recently become available in some countries. The 10-valent vaccine includes tetanus toxoid, diphtheria toxoid or a novel protein derived from non-typable Haemophilus influenzae (protein D) as the carrier proteins, while the 13-valent vaccine uses only CRM197 as the carrier protein.

Vaccine efficacy against IPD has been evaluated in randomized and controlled studies in children aged less than 2 years. The studies employed the 7vPnC vaccine or an experimental 9vPnC vaccine that included all seven serotypes in the 7vPnC vaccine. At the time that these studies were initiated, no licensed pneumococcal conjugate vaccine was available; control groups therefore did not receive a pneumococcal conjugate vaccine. The studies provided data from the Gambia (12), South Africa (13) and the USA (for the general population and for native American children) (7, 8, 14). The 7vPnC vaccine and the 9vPnC vaccine were shown to be efficacious in preventing IPD, although serotype-specific efficacy could be estimated for only four of the serotypes.

Post-marketing effectiveness data from countries in which the 7vPnC vaccine has been introduced into the routine infant and toddler immunization programmes have shown a reduction in rates of IPD in children aged less than 2 years due to all seven vaccine serotypes and also to serotype 6A, which is not included in the vaccine (7, 15). In addition, routine use of the 7vPnC vaccine in infants and toddlers has been associated with reduced rates of IPD in the elderly population, indicating that there is an indirect beneficial effect (i.e. a herd immunity effect) in unvaccinated persons (15). Correspondingly, studies have demonstrated that the 7vPnC vaccine reduces rates of nasopharyngeal carriage of serotypes included in the vaccine and of some types that are not included. Thus far, the safety profile of 7vPnC vaccine is considered to be acceptable (9, 10, 16, 17).

WHO Recommendations for pneumococcal conjugate vaccine production and control were first established in 2003 and published in the WHO Technical Report Series (TRS 927, Annex 2). In that document, it was considered that practical or ethical considerations might make it impossible to perform protective efficacy trials (i.e. using an unvaccinated control group). The recommendations therefore covered the design of immunogenicity studies necessary to support the licensing of new pneumococcal conjugate vaccines (including those containing conjugated capsular polysaccharides of serotypes additional to those in the 7vPnC vaccine) intended to prevent IPD and for administration to children aged less than 2 years.
It was considered essential that the immunogenicity studies with a new pneumococcal conjugate vaccine should provide a link back to the efficacy against IPD that was demonstrated for the 7vPnC vaccine. Thus, it was recommended that immune responses to each serotype in the 7vPnC vaccine that is also included in a new pneumococcal conjugate vaccine should be directly compared in randomized clinical studies and that the primary comparison of immune responses should be based on serotype-specific IgG antibody concentrations measured by enzyme-linked immunosorbent assay (ELISA). In order to facilitate these comparisons, a WHO reference ELISA assay was established that includes pre-adsorption of sera with pneumococcal C polysaccharide (C-PS) and serotype 22F polysaccharide. Appendix 1 of this document explains these pre-adsorption steps and provides details of the validation, standardization and bridging of ELISA assays.

The immunogenicity data and estimates of vaccine efficacy against IPD across all serotypes in the 7vPnC vaccine were pooled for three of the above-mentioned randomized, controlled efficacy studies in infants and toddlers (see Table A3.1). Serum concentrations of IgG were measured using a well-characterized ELISA method (which differed from the WHO reference ELISA only in that it did not include a 22F adsorption step). For this particular ELISA protocol, it was subsequently shown that the pre-absorption of sera with C-PS and 22F had a minimal effect on estimations of IgG concentrations in a selection of sera from infants who received 7vPnC or 9vPnC vaccines (18). On the basis of these data, an IgG antibody concentration of 0.35 μg/ml (assessed using the WHO ELISA) was suggested as a benchmark (or threshold value) for comparing immune responses to each serotype common to the 7vPnC vaccine and a new pneumococcal conjugate vaccine (19). The rationale for selecting this threshold antibody concentration is described in more detail in the report of a WHO meeting (20). Briefly, results from three clinical trials (Table A3.1) were pooled to derive the threshold value of 0.35 μg/ml. The numbers of IPD cases in the vaccinated and unvaccinated cohorts of each trial, as well as the total number of participants, were summed and used to calculate a pooled estimate of 93% for vaccine efficacy. This vaccine efficacy was then referred to a pooled reverse cumulative distribution (RCD) curve to derive the final 0.35 μg/ml threshold (18). Thus, this value is not an average estimate using the trial-specific thresholds listed in Table A3.1 but is derived from the pooled RCD curve.

While this population-derived IgG antibody threshold value is considered to be a useful “benchmark”, it is important that it is not interpreted to mean that achievement of ≥ 0.35 μg/ml for a specific serotype (whether included in the 7vPnC vaccine or in a new pneumococcal conjugate vaccine) predicts protection of an individual against PD due to that serotype.
It was recognized that a threshold based on opsonophagocytic assay (OPA) titres (which reflect functional antibody) might also be suitable for comparing immune responses between vaccines, and it was recommended that OPA data should be generated for a subset of vaccinated subjects in clinical studies. The limited data obtained during the protective efficacy studies conducted with the 7vPnC vaccine indicated that an IgG concentration ≥0.2 µg/ml (determined without 22F pre-adsorption of sera) corresponded approximately to an OPA titre ≥1:8 for some serotypes (20). Methods for determining OPA are also discussed in Appendix 1.

Prompted by issues raised during the development of newer pneumococcal conjugate vaccines since the publication of TRS 927 in 2003, WHO held a consultation in 2008 (21) to consider new scientific evidence and discuss the need to provide revised guidance for manufacturers and licensing authorities. Inter alia, the consultation reviewed effectiveness data obtained with various immunization schedules for the 7vPnC vaccine in Canada (22), the United Kingdom (23) and the USA (7). Technical developments in ELISA and OPA methods, variability between assays and the need for standardization were considered. The importance of bridging new assays to the WHO reference ELISA method when determining IgG concentrations was discussed, along with the option of establishing an assay-specific alternative threshold value to ≥0.35 µg/ml.
During the 2008 consultation, some data were provided that supported the use of the IgG antibody threshold as a benchmark value. For example, data from the United Kingdom had shown that only 30–50% of infants reached the threshold of ≥ 0.35 µg/ml against 6B after two doses of 7vPnC vaccine at 2 and 4 months of age and that this was associated with vaccine failures due to 6B in the interval between the second dose and the third dose at 13 months. However, previous and newer data suggested that IgG antibody concentrations below 0.35 µg/ml may be sufficient to prevent IPD due to some serotypes. In addition, some data suggested that OPA titres against certain serotypes (e.g. 19A) correlated better with estimates of effectiveness than IgG concentrations when measured using the WHO reference assay (24).

Overall it was considered that some of the information accrued since 2003 merited incorporation into updated WHO Recommendations for pneumococcal conjugate vaccines. Most of the revisions pertain to the clinical assessment of new pneumococcal vaccines.

**Part A. Manufacturing recommendations**

A.1 **Definitions**

A.1.1 **Proper name**

The proper name of the vaccine shall be “pneumococcal conjugate vaccine” translated into the language of the country of use. The serotypes included in the vaccine should be associated with the name of the vaccine and listed in the packaging material. The use of this proper name should be limited to vaccines that satisfy the specifications formulated below.

A.1.2 **Descriptive definition**

Multivalent pneumococcal conjugate vaccine is a preparation of capsular polysaccharide from specific serotypes of *Streptococcus pneumoniae* that are covalently linked to carrier protein.

A.1.3 **International Reference Materials**

No formally established International Reference Materials that would allow the standardization of immune responses to pneumococcal conjugate vaccines are currently available.

The following reagents are available through the courtesy of individuals, manufacturers and national control or reference laboratories:

- C-polysaccharide (Statens Serum Institut, Copenhagen, Denmark);
- capsular polysaccharides (American Type Culture Collection, Manassas, VA, USA);
- 89-SF reference serum (Center for Biologics Evaluation and Research, Washington, DC, USA);
- 96DG secondary reference serum (provided by Dr David Goldblatt and distributed by National Institute for Biological Standardization and Control, Potters Bar, England);
- ELISA calibration sera (provided by Dr David Goldblatt and distributed by National Institute for Biological Standardization and Control, Potters Bar, England);
- pneumococcal serotyping reagents (Statens Serum Institut, Copenhagen, Denmark);
- HL-60 cells (American Type Culture Collection, Manassas, VA, USA or European Collection of Cell Cultures, Porton Down, Salisbury, England).

A.1.4 **Terminology**

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

**Master seed lot.** A bacterial suspension of *Streptococcus pneumoniae* derived from a strain that has been processed as a single lot and is of uniform composition. It is used for the preparation of the working seed lots. Master seed lots shall be maintained in the freeze-dried form or be frozen below –45 °C.

**Working seed lot.** A quantity of live *Streptococcus pneumoniae* organisms derived from the master seed lot by growing the organisms and maintaining them in aliquots in the freeze-dried form or frozen state at or below –45 °C. The working seed lot is used, when applicable, after a fixed number of passages, for the inoculation of production medium.

**Single harvest.** The material obtained from one batch of cultures that have been inoculated with the working seed lot (or with the inoculum derived from it), harvested and processed together.

**Purified polysaccharide.** The material obtained after final purification. The lot of purified polysaccharide may be derived from a single harvest or a pool of single harvests processed together.

**Modified polysaccharide.** Purified polysaccharide that has been modified by chemical reaction or physical process in preparation for conjugation to the carrier.

**Carrier.** The protein to which the polysaccharide is covalently linked for the purpose of eliciting a T-cell-dependent immune response to the pneumococcal polysaccharide.

**Monovalent bulk conjugate.** A conjugate prepared from a single lot or pool of lots of polysaccharide and a single lot or a pool of lots of protein. This is the parent material from which the final bulk is prepared.
**Final bulk conjugate.** The blend of monovalent conjugates present in a single container from which the final containers are filled, either directly or through one or more intermediate containers derived from the initial single container.

**Final lot.** A number of sealed, final containers that are equivalent with respect to the risk of contamination during filling and, when it is performed, freeze-drying. A final lot must therefore have been filled from a single container and freeze-dried in one continuous working session.

### A.2 General manufacturing requirements

The general manufacturing recommendations contained in Good manufacturing practices for pharmaceutical products (25) and Good manufacturing practices for biological products (26) should be applied to establishments manufacturing pneumococcal conjugate vaccines with the addition of the following:

Details of standard operating procedures for the preparation and testing of pneumococcal conjugate vaccines adopted by the manufacturer, together with evidence of appropriate validation of each production step, should be submitted for the approval of the national regulatory authority (NRA). All assay procedures used for quality control of the conjugate vaccines and vaccine intermediates must be validated. As may be required, proposals for the modification of manufacturing and control methods should also be submitted for approval to the NRA before they are implemented.

*Streptococcus pneumoniae* is a Biosafety Level 2 (BSL-2) pathogen and represents a particular hazard to health through infection by the respiratory route. The organism should be handled under appropriate conditions for this class of pathogen (27). Standard operating procedures need to be developed for dealing with emergencies arising from the accidental spillage, leakage or other dissemination of pneumococcal organisms. Personnel employed in the production and control facilities should be adequately trained and appropriate protective measures, including vaccination with a pneumococcal vaccine licensed for use in adults, should be implemented. Adherence to current good manufacturing practices is important to the integrity of the product, to protect workers and to protect the environment.

### A.3 Production control

#### A.3.1 Control of polysaccharide

#### A.3.1.1 Strains of *Streptococcus pneumoniae*

The strains of *S. pneumoniae* used for preparing the polysaccharide should be agreed with the NRA. Each strain should have been shown to be capable of
producing polysaccharide of the appropriate serotype. Each master seed lot should be identified by a record of its history, including the source from which it was obtained and the tests made to determine the characteristics of the strain.

The cultures may be examined for the following characteristics: microscopically, stained smears from a culture should appear typical of \textit{S. pneumoniae}; the organism should grow at 37 °C but not at 25 °C, and should have characteristic smooth alpha-haemolytic colonies; the organism should have the ability to ferment inulin; the organism should be lysed in the bile solubility test and be sensitive to optochin; a suspension of the culture should be agglutinated or give a positive Quellung reaction with the appropriate serotyping serum.

Nuclear magnetic resonance (NMR) spectroscopy (either $^1$H or $^{13}$C) is a suitable method for the confirmation of identity of purified polysaccharide.

A.3.1.2 Seed lot system

The production of pneumococcal polysaccharide should be based on a working seed lot system. Cultures derived from the working seed lots should have the same characteristics as the cultures of the strain from which the master seed lot was derived (A.3.1.1). If materials of animal origin are used in the medium for seed production, for preservation of strain viability for freeze-drying or for frozen storage, they should comply with \textit{WHO guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products} (28) and should be approved by the NRA.

Wherever possible, manufacturers are encouraged to avoid the use of materials of animal origin.

A.3.1.3 Culture media for the production of pneumococcal polysaccharide

The liquid culture medium used for vaccine production should be free from ingredients that will form a precipitate upon purification of the capsular polysaccharide. If materials of animal origin are used, they should comply with \textit{WHO guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products} (28) and should be approved by the NRA.

Wherever possible, manufacturers are encouraged to avoid the use of materials of animal origin.

A.3.1.4 Single harvests

Consistency of growth of \textit{S. pneumoniae} should be demonstrated by monitoring growth rate, pH and the final yield of polysaccharide.
A.3.1.5  **Control of bacterial purity**

Samples of the culture should be taken before killing and be examined for microbial contamination. The purity of the culture should be verified by suitable methods, which should include inoculation on to appropriate culture media, including plate media that do not support growth of *S. pneumoniae*. If any contamination is found, the culture or any product derived from it should be discarded. The killing process should also be adequately validated.

A.3.1.6  **Purified polysaccharide**

Each lot of pneumococcal polysaccharide should be tested for identity, purity and molecular size. A number of approaches to determining polysaccharide identity and purity give complementary but incomplete information, so a combination of methods should be employed to provide all necessary data and should be agreed by the NRA. The purity limits given below are expressed with reference to the polysaccharide in its salt form (sodium or calcium), corrected for moisture. Variations in these specifications that may be appropriate if unusual salt forms are present should be agreed by the NRA.

Generally, after the organism is killed, the culture is harvested and the polysaccharide isolated and purified by techniques such as fractional precipitation, chromatography, enzyme treatment and ultrafiltration. The polysaccharide is partially purified by fractional precipitation, washed and dried to a residual moisture content shown to favour its stability. Methods used for the purification of bulk polysaccharide should be approved by the NRA. Purified pneumococcal polysaccharide and, when necessary, partially purified intermediates are usually stored at or below −20 °C to ensure stability.

A.3.1.6.1  **Polysaccharide identity**

A test should be performed on the purified polysaccharide to verify its identity. In cases where other polysaccharides are produced on the same manufacturing site, the method should be validated to show that it distinguishes the desired polysaccharide from all other polysaccharides produced on that manufacturing site.

A serological method such as countercurrent immuno-electrophoresis and/or NMR spectroscopy (either $^1$H or $^{13}$C) is convenient for this purpose (29–31). In some cases, if appropriate analytical methods are employed, the identity of the polysaccharide can be deduced from its composition.

A.3.1.6.2  **Polysaccharide composition**

The composition of the polysaccharide provides information on its purity, identity and the amounts of specific impurities, such as pneumococcal C-polysaccharide,
that are present. Analyses should be based on the dry weight of the polysaccharide. The composition of the polysaccharide can be defined in a number of ways depending on the methodology employed and the salt form present (Table A3.2). The specifications used should be agreed by the NRA.

Chemically, the composition of pneumococcal polysaccharides can be defined by the percentage of total nitrogen, phosphorus, uronic acid, hexosamine, methyl pentose and \(O\)-acetyl groups. These are usually determined by a combination of simple wet chemical tests with colorimetric read outs. Typical specifications are tabulated below (32); they may be adapted when other methods such as \(^1\)H-NMR are used.

Other methods, such as high-performance anion exchange chromatography (HPAEC) with electrochemical detection, with pulsed amperometric detection (HPAEC-PAD) applied to hydrolysates of the polysaccharide, may be used to define aspects of the quantitative composition of certain polysaccharide types, but the method should be validated for the purpose (33). NMR spectroscopy \((\text{\(^1\)H})\) is also a convenient means of quantitatively defining the composition of the purified polysaccharide if an internal reference compound is included (30, 31). The proportion of pneumococcal C polysaccharide may be determined by a combination of \(^1\)H and \(^31\)P NMR spectroscopy (34, 35) or HPAEC-PAD (36).

A.3.1.6.3 **Moisture content**

If the purified polysaccharide is to be stored as a lyophilized powder, the moisture content should be determined by suitable methods approved by the NRA and shown to be within agreed limits.

A.3.1.6.4 **Protein impurity**

The protein content should be determined by the method of Lowry et al., using bovine serum albumin as a reference (37), or another suitable validated method. Sufficient polysaccharide should be assayed to detect 1% protein contamination accurately.

Each lot of purified polysaccharide should typically contain not more than 3% by weight of protein. However, this will vary depending upon the serotype, and an acceptable level of protein contamination should be agreed with the NRA.

A.3.1.6.5 **Nucleic acid impurity**

Each lot of polysaccharide should contain not more than 2% by weight of nucleic acid as determined by ultraviolet spectroscopy – on the assumption that the
absorbance of a 1 g/l nucleic acid solution contained in a cell of 1 cm path length at 260 nm is 20 (38) – or by another validated method.

Sufficient polysaccharide shall be assayed to detect 2% nucleic acid contamination accurately.

A.3.1.6.6  **Pyrogen content**

The pyrogen content of the purified polysaccharide should be determined and shown to be within acceptable limits agreed by the NRA.

A recognized pyrogenicity test can be performed in rabbits; alternatively, the *Limulus* amoebocyte lysate test can be performed.

A.3.1.6.7  **Molecular size distribution**

The molecular size of each lot of purified polysaccharide provides an indication of the manufacturing consistency. An acceptable level of consistency should be agreed with the NRA and can be established either by process validation or by measurement on each lot.

The distribution constant ($K_D$) can be determined by measuring the molecular size distribution of the polysaccharide at the main peak of the elution curve obtained by a suitable chromatographic method. The $K_D$ value and/or the mass distribution limits should be established.

Methods suitable for this purpose include: gel filtration through Sepharose CL-4B or CL-6B (or similar) in a 0.2 M buffer using either a refractive index detector or colorimetric assay for detection of the polysaccharide; and high-performance size-exclusion chromatography (HPSEC) with refractive index detectors either alone or in combination with light scattering (e.g. multiple-angle laser light scattering, MALLS) (31, 39). The methodology and column used should be validated to demonstrate sufficient resolution in the appropriate molecular weight range.

A.3.1.7  **Modified polysaccharide**

Modified polysaccharide preparations may be partially depolymerized either before or during the chemical modification. Pneumococcal conjugate vaccines use polysaccharides and oligosaccharide chains.

A.3.1.7.1  **Chemical modification**

Several methods for the chemical modification of polysaccharides prior to conjugation may be satisfactory. The chosen method should be approved by the NRA.
The methods used currently are similar to those employed in the production of conjugate vaccines against *Haemophilus influenzae* type b. For example, polysaccharide may be oxidized with periodate and the periodate-activated polysaccharide attached to free amino groups on the carrier protein by reductive amination. Alternatively, the polysaccharide can be randomly activated by cyanogen bromide, or a chemically similar reagent; a bifunctional linker is added, which then allows the polysaccharide to be attached to the carrier protein either directly or through a secondary linker.

A.3.1.7.2 *Extent of modification of the polysaccharide*

The manufacturer should demonstrate consistency of the degree of modification of the polysaccharide, either by an assay of each batch of the polysaccharide or by validation of the manufacturing process. Depending on the conjugation chemistry used, consistency in degree of polysaccharide activation may be determined as part of process validation or reflected by characteristics of vaccine lots shown to have adequate safety and immunogenicity in clinical trials.

A.3.1.7.3 *Molecular size distribution*

The degree of size reduction of the polysaccharide will depend upon the manufacturing process. The average size distribution (degree of polymerization) of the modified polysaccharide should be determined by a suitable method and shown to be consistent. The molecular size distribution should be specified for each serotype, with appropriate limits for consistency, as the size may affect the reproducibility of the conjugation process.

The molecular size may be determined by gel filtration on soft columns or by HPSEC using refractive index alone, or in combination with laser light scattering (e.g. MALLS) (31, 39). An alternative method shown to correlate to molecular size distribution (e.g. measurement of viscosity) may be used to show consistency with size reduction of the polysaccharide.
Table A3.2
Theoretical composition of pneumococcal polysaccharides

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Total nitrogen (%) (range)</th>
<th>Phosphorus&lt;sup&gt;a&lt;/sup&gt; (%) (range)</th>
<th>Uronic acid (%)</th>
<th>Hexosamines (%)</th>
<th>Methyl pentose (%)</th>
<th>O-acetyl groups (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.56 (3.5–6)</td>
<td>0 (0–1.5)</td>
<td>55.17 (≥45)</td>
<td>0</td>
<td>0</td>
<td>5.47 (≥1.8)</td>
</tr>
<tr>
<td>2</td>
<td>0 (0–1)</td>
<td>0 (0–1.0)</td>
<td>22.59 (≥5)</td>
<td>0</td>
<td>50.58 (≥38)</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0 (0–1)</td>
<td>0 (0–1.0)</td>
<td>60.23 (≥40)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>4.95 (4–6)</td>
<td>0 (0–1.5)</td>
<td>0</td>
<td>71.84 (≥40)</td>
<td>19.11 (≥10)</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>3.04 (2.5–6)</td>
<td>0 (&lt;2)</td>
<td>23.59 (≥12)</td>
<td>44.14 (≥20)</td>
<td>35.22 (≥25)</td>
<td>0</td>
</tr>
<tr>
<td>6B</td>
<td>0 (0–2)</td>
<td>4.38 (2.5–5.0)</td>
<td>0</td>
<td>0</td>
<td>22.86 (≥15)</td>
<td>0</td>
</tr>
<tr>
<td>7F</td>
<td>2.28 (1.5–4.0)</td>
<td>0 (0–1.0)</td>
<td>0</td>
<td>33.09</td>
<td>26.40 (≥13)</td>
<td>3.5 (present)</td>
</tr>
<tr>
<td>8</td>
<td>0 (0–1)</td>
<td>0 (0–1.0)</td>
<td>31.70 (≥25)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9N</td>
<td>3.09 (2.2–4.0)</td>
<td>0 (0–1.0)</td>
<td>23.96 (≥20)</td>
<td>44.82 (≥28)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9V</td>
<td>1.44 (0.5–3)</td>
<td>0 (0–1.0)</td>
<td>22.33 (≥15)</td>
<td>20.89 (≥13)</td>
<td>0</td>
<td>8.85 (present)</td>
</tr>
<tr>
<td>10A</td>
<td>1.12 (0.5–3.5)</td>
<td>2.48 (1.5–3.5)</td>
<td>0</td>
<td>16.21 (≥12)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>11A</td>
<td>0 (0–2.5)</td>
<td>3.25 (2.0–5.0)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>13.54 (≥9)</td>
</tr>
<tr>
<td>12F</td>
<td>3.82 (3–5)</td>
<td>0 (0–1.0)</td>
<td>19.73 (≥15)</td>
<td>55.36 (≥25)</td>
<td>14.73 (≥10)</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>2.03 (1.5–4)</td>
<td>0 (0–1.0)</td>
<td>0</td>
<td>29.44 (≥20)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>15B</td>
<td>1.31 (1–3)</td>
<td>2.89 (2.0–4.5)</td>
<td>0</td>
<td>18.94 (≥15)</td>
<td>0</td>
<td>4.01 (present)</td>
</tr>
<tr>
<td>17A</td>
<td>0 (0–1.5)</td>
<td>0 (0–3.5)</td>
<td>16.16 (≥10)</td>
<td>0</td>
<td>24.12 (≥20)</td>
<td>3.2 (present)</td>
</tr>
</tbody>
</table>

continues
### Table A3.2 continued

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Total nitrogen (%) (range)</th>
<th>Phosphorus(^a) (%) (range)</th>
<th>Uronic acid (%)</th>
<th>Hexosamines (%)</th>
<th>Methyl pentose (%)</th>
<th>O-acetyl groups (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17F</td>
<td>0 (0–1.5)</td>
<td>2.93 (0–3.5)</td>
<td>0</td>
<td>0</td>
<td>30.60 (≥20)</td>
<td>4.06 (present)</td>
</tr>
<tr>
<td>18C</td>
<td>0 (0–1)</td>
<td>3.05 (2.4–4.9)</td>
<td>0</td>
<td>0</td>
<td>15.96 (≥14)</td>
<td>4.24 (present)</td>
</tr>
<tr>
<td>19A</td>
<td>2.27 (0.6–3.5)</td>
<td>5.04 (3.0–7.0)</td>
<td>0</td>
<td>32.98 (≥12)</td>
<td>26.32 (≥20)</td>
<td>0</td>
</tr>
<tr>
<td>19F</td>
<td>2.27 (1.4–3.5)</td>
<td>5.04 (3.0–5.5)</td>
<td>0</td>
<td>32.98 (≥12.5)</td>
<td>26.32 (≥20)</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>1.28 (0.5–2.5)</td>
<td>0 (1.5–4.0)</td>
<td>0</td>
<td>18.49 (≥12)</td>
<td>0</td>
<td>7.83 (present)</td>
</tr>
<tr>
<td>22F</td>
<td>0 (0–2)</td>
<td>0 (0–1.0)</td>
<td>21.30 (≥15)</td>
<td>0</td>
<td>31.80 (≥25)</td>
<td>4.22 (present)</td>
</tr>
<tr>
<td>23F</td>
<td>0 (0–1)</td>
<td>3.90 (3.0–4.5)</td>
<td>0</td>
<td>40.77 (≥37)</td>
<td>0</td>
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</tr>
<tr>
<td>33F</td>
<td>0 (0–2)</td>
<td>0 (0–1.0)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4.24 (present)</td>
</tr>
</tbody>
</table>

\(^a\) Theoretical value with suggested range in parentheses, based on published structures. These are calculated using broad definitions of the classes of sugars; so, for example “hexosamine” includes 2-acetamido-2,6-dideoxyhexoses and 2-acetamido-2-deoxyuronic acids, “methylpentose” includes 2-acetamido-2,6-dideoxyhexoses, and “uronic acid” includes 2-acetamido-2-deoxyuronic acids. It is not certain that such sugars would give an identical response in chemical tests used to determine the composition. The values are cited as equivalents of probable reference compounds used in such compositional tests. The values assume complete O-acetylation at each distinct site for O-acetylation, using published and unpublished data.
A.3.2 Control of the carrier protein

A.3.2.1 Microorganisms and culture media for production of carrier protein

Microorganisms to be used for the production of the carrier protein should be grown in media free from substances likely to cause toxic or allergic reactions in humans. If any materials of animal origin are used in seed preparation or preservation or in production, they should comply with the WHO guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products (28) and should be approved by the NRA.

Production should be based on a seed lot system, with the strains identified by a record of their history and of all tests made periodically to verify strain characteristics. Consistency of growth of the microorganisms used should be demonstrated by monitoring the growth rate, pH and final yield of appropriate protein(s).

A.3.2.2 Characterization and purity of the carrier protein

There are many proteins that could potentially be used as carriers in pneumococcal conjugate vaccines. The principal characteristics of the carrier protein should be that it is safe and, in the conjugate, elicits a T-cell-dependent immune response against the polysaccharide. Test methods used to characterize such proteins, to ensure that they are non-toxic and to determine their purity and concentration, should be approved by the NRA.

Proteins and purification methods that might be used include:

**Tetanus or diphtheria toxoid.** This must satisfy the relevant Requirements published by WHO (40) and be of high purity (41).

**Diphtheria CRM 197 protein.** This is a non-toxic mutant of diphtheria toxin, isolated from cultures of Corynebacterium diphtheriae C7/β197 (42). Protein purity should be greater than 90% as determined by an appropriate method. When produced in the same facility as diphtheria toxin, methods must be in place to distinguish the CRM 197 protein from the active toxin.

**Protein D derived from non-typable Haemophilus influenzae.** The routine release should include tests to confirm identity and purity of the protein as approved by the NRA, supplemented by additional data to characterize the protein.

The protein carrier should also be characterized. The identity may be determined serologically. Physicochemical methods that may be used to characterize protein include sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE), isoelectric focusing, high-performance liquid chromatography (HPLC), amino acid analysis, amino acid sequencing, circular dichroism, fluorescence spectroscopy, peptide mapping and mass spectrometry as appropriate (31).
A.3.3  Control of monovalent bulk conjugates

There are a number of possible conjugation methods that might be used for vaccine manufacture; all involve multi-step processes. Both the method and the control procedures used to ensure the reproducibility, stability and safety of the conjugate should be established for licensing. The derivatization and conjugation process should be monitored by analysis for unique reaction products or by other suitable means. The conditions used in the conjugation chemistry may affect the structure of the polysaccharide chain by causing the loss of labile substituents. Unless the results of the tests used to characterize the bulk monovalent conjugate can provide information on structural changes, an explicit identity test on the polysaccharide present should be performed.

Residual activated functional groups potentially capable of reacting in vivo may be present following the conjugation process. The manufacturing process should be validated to show that no activated functional groups remain at the conclusion of the manufacturing process or that the level of any remaining groups is below a limit approved by the NRA.

After the conjugate has been purified, the tests described below are usually performed on non-adsorbed conjugate bulks. Alternatively, they may be performed on adsorbed monovalent conjugate bulks, e.g. in case individual conjugate bulks are adsorbed to adjuvant before final formulation of the vaccine. The tests are critical for assuring lot-to-lot consistency.

A.3.3.1  Identity

A test should be performed on the monovalent bulk to verify its identity. The method should be validated to show that it distinguishes the desired monovalent material from all other polysaccharides and conjugates produced on that manufacturing site.

A.3.3.2  Residual reagents

The conjugate purification procedures should remove residual reagents used for conjugation and capping. The removal of reagents and reaction by products such as cyanide, 1-ethyl-3,3-(3-dimethylaminopropyl)-carbodiimide (EDAC) and others, depending on the conjugation chemistry, should be confirmed by suitable tests or by validation of the purification process.

The residuals are process-specific and can be quantified by use of colorimetric and chromatographic assays. Techniques such as NMR spectroscopy and hyphenated techniques such as liquid chromatography–mass spectrometry may also be applied.
A.3.3 Polysaccharide–protein ratio and conjugation markers

For each batch of the bulk conjugate of each serotype, the ratio of polysaccharide to carrier protein should be determined as a marker of the consistency of the conjugation chemistry. For each conjugate, the ratio should be within the range approved for that particular conjugate by the NRA and should be consistent with vaccine shown to be effective in clinical trials.

For pneumococcal conjugate vaccines the ratio is typically in the range 0.3–3.0 but varies with the serotype. The ratio can be determined either by independent measurement of the amounts of protein and polysaccharide present, or by methods that give a direct measure of the ratio. Methods include 1H NMR spectroscopy or the use of HPSEC with dual monitoring (e.g. refractive index and UV, for total material and protein content respectively).

If the chemistry of conjugation results in the creation of a unique linkage marker (e.g. a unique amino acid), each batch of the bulk conjugate of that serotype should be assessed to quantify the extent of substitution of the carrier protein by covalent reaction of the pneumococcal polysaccharide with the carrier protein.

The structural complexity and structural differences between the pneumococcal serotypes are such that in most cases it will not be possible to identify a simple conjugation marker.

A.3.4 Capping markers

Each batch should be shown to be free of activated functional groups on either the chemically modified polysaccharide or the carrier protein. Alternatively, the product of the capping reaction can be monitored or the capping reaction can be validated to show removal of unreacted functional groups. Validation of the manufacturing process during vaccine development can eliminate the need to perform this analysis for routine control.

A.3.5 Conjugated and unbound (free) polysaccharide

Only the pneumococcal polysaccharide that is covalently bound to the carrier protein, i.e. conjugated polysaccharide, is immunologically important for clinical protection. Each batch of conjugate should be tested for unbound or free polysaccharide in order to establish consistency of production and to ensure that the amount present in the purified bulk is within the limits agreed by the NRA based on lots shown to be clinically safe and efficacious.

Methods that have been used to separate unbound polysaccharide before assay, and that are potentially applicable to pneumococcal conjugates,
include hydrophobic chromatography, acid precipitation, precipitation with carrier protein-specific antibodies, gel filtration and ultrafiltration. The amount of unbound polysaccharide can be determined by specific chemical or immunological tests, or by HPAEC after hydrolysis.

A.3.3.6 Protein content

The protein content of the conjugate should be determined by means of an appropriate validated assay and comply with limits for the particular product. Each batch should be tested for conjugated and unbound protein.

If possible, the unconjugated protein should also be measured. Appropriate methods for the determination of conjugated and unconjugated protein include HPLC and capillary electrophoresis.

A.3.3.7 Molecular size distribution

The molecular size of the polysaccharide–protein conjugate is an important parameter in establishing consistency of production and in studying stability during storage.

The relative molecular size of the polysaccharide–protein conjugate should be determined for each bulk, using a gel matrix appropriate to the size of the conjugate. The method should be validated with an emphasis on specificity to distinguish the polysaccharide–protein conjugate from other components that may be present, e.g. unbound protein or polysaccharide. The size distribution specifications will be vaccine-specific and should be consistent with lots shown to be immunogenic in clinical trials.

Typically the size may be examined by gel filtration on Sepharose CL-2B or by HPSEC on an appropriate column. Since the polysaccharide–protein ratio is an average value, characterization of this ratio over the size distribution (e.g. by dual monitoring of the column eluent) can be used to provide further proof of manufacturing consistency (43).

A.3.3.8 Sterility

The bulk purified conjugate should be tested for bacterial and mycotic sterility in accordance with the requirements of Part A, sections 5.1 and 5.2, of the revised General requirements for the sterility of biological substances (44) or by a method approved by the NRA. If a preservative has been added to the product, appropriate measures should be taken to prevent it from interfering with the test.

A.3.3.9 Specific toxicity of carrier protein

The bulk conjugate should be tested for the absence of specific toxicity of the carrier protein where appropriate (e.g. when tetanus or diphtheria toxoids have
been used). Absence of specific toxicity of the carrier protein may also be assessed through validation of the production process.

A.3.3.10 **Endotoxin content**

To ensure an acceptable level of endotoxin in the final product, the endotoxin content of the monovalent bulk may be determined and shown to be within acceptable limits agreed by the NRA.

A.3.4 **Final bulk**

A.3.4.1 **Preparation**

To formulate the final bulk, monovalent conjugate bulks may be mixed together and an adjuvant, preservative and/or stabilizer added before final dilution. Alternatively, the monovalent conjugate bulks may be adsorbed to adjuvant individually before mixing them to formulate the final vaccine.

A.3.4.2 **Sterility**

Each final bulk should be tested for bacterial and mycotic sterility as indicated in section A.3.3.8.

A.3.5 **Filling and containers**

The recommendations concerning filling and containers given in Good manufacturing practices for biological products should be applied (26).

A.3.6 **Control tests on final product**

A.3.6.1 **Identity**

An identity test should be performed that demonstrates that all of the intended pneumococcal polysaccharide serotypes and carrier protein(s) are present in the final product, unless this test has been performed on the final bulk.

A serological test, using antibodies specific for the purified polysaccharide may be used.

A.3.6.2 **Sterility**

The contents of final containers should be tested for bacterial and mycotic sterility as indicated in section A.3.3.8.

A.3.6.3 **Pneumococcal polysaccharide content**

The amount of each pneumococcal polysaccharide in the final containers should be determined and shown to be within the specifications agreed by the NRA.
The conjugate vaccines produced by different manufacturers differ in formulation. A quantitative assay should be carried out for each of the pneumococcal polysaccharides in the final container. The assays used are likely to be product-specific and might include chromatographic or serological methods. Immunological assays such as rate nephelometry (45) or ELISA inhibition may be used.

Assessment of the content of each serotype in the final vaccine may be difficult and may require complex methodologies not available to national control laboratories (NCLs). In the event that testing is performed in the framework of lot release by NCLs, measurement of the total polysaccharide content could therefore be authorized.

A.3.6.4 Residual moisture

If the vaccine is freeze-dried, the average moisture content should be determined by methods accepted by the NRA. Values should be within the limits for the preparations shown to be adequately stable in the stability studies of the vaccine.

The test should be performed on 1 vial per 1000 up to a maximum of 10 vials but on no less than 5 vials taken at random from throughout the final lot. The average residual moisture content should generally be no greater than 2.5% and no vial should be found to have a residual moisture content of 3% or greater.

A.3.6.5 Endotoxin content

The vaccine in the final container should be tested for endotoxin content by a *Limulus* amoebocyte lysate test (LAL). 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.

A.3.6.6 Adjuvant content

If an adjuvant has been added to the vaccine, its content should be determined by a method approved by the NRA. The amount and nature of the adjuvant should be agreed with the NRA. If aluminium compounds are used as adjuvants, the amount of aluminium should not exceed 1.25 mg per single human dose.

A.3.6.7 Preservative content

The manufacturer has a choice of possible preservatives. Consideration should be given to the stability of the chosen preservative and possible interactions between the vaccine components and the preservative. If a preservative has been added to the vaccine, the content of preservative should be determined by a method approved by the NRA. The amount of preservative in the vaccine dose should be shown not to have any deleterious effect on the antigen or to impair the safety
of the product in humans. The preservative and its concentration should be approved by the NRA.

A.3.6.8 General safety test (innocuity)
The requirement to test lots of pneumococcal conjugate vaccine for unexpected toxicity (abnormal toxicity) should be agreed with the NRA.

Such a test may be omitted for routine lot release once consistency of production has been well established to the satisfaction of the NRA and when good manufacturing practice is in place.

A.3.6.9 pH
If the vaccine is a liquid preparation, the pH of each final lot should be tested and shown to be within the range of values found for vaccine lots shown to be safe and effective in clinical trials and in stability studies. For a lyophilized preparation, the pH should be measured after reconstitution with the appropriate diluent.

A.3.6.10 Inspection of final containers
Each container in each final lot should be inspected visually (manually or with automatic inspection systems), and those showing abnormalities such as improper sealing, lack of integrity and, if applicable, clumping or the presence of particles should be discarded.

A.4 Records
The recommendations in section 8 of Good manufacturing practices for biological products (26) should be applied.

A.5 Retained samples
The recommendations in section 9.5 of Good manufacturing practices for biological products (26) should be applied.

A.6 Labelling
The recommendations in section 7 of Good manufacturing practices for biological products (26) should be applied with the addition of the following:

The label on the carton or the leaflet accompanying the container should indicate:

- the pneumococcal serotype and carrier protein present in each single human dose;
- the amount of each conjugate present in a single human dose;
- the temperature recommended during storage and transport;
- if the vaccine is freeze-dried, that after its reconstitution it should be used immediately unless data have been provided to the licensing authority showing that it may be stored for a limited time;
- the volume and nature of the diluent to be added in order to reconstitute a freeze-dried vaccine, specifying that the diluent should be supplied by the manufacturer and approved by the NRA.

A.7  Distribution and transport
The recommendations in section 8 of Good manufacturing practices for biological products (26) should be applied.

A.8  Stability, storage and expiry date
A.8.1  Stability testing
Adequate stability studies form an essential part of the vaccine development studies. These studies should follow the general principles outlined in Guidelines on stability evaluation of vaccines (46). The stability of the vaccine in its final form and at the recommended storage temperatures should be demonstrated to the satisfaction of the NRA with final containers from at least three lots of final product made from different independent bulk conjugates.

Given the complexity of these multivalent vaccines, other approaches may be used with the approval of the NRA.

The polysaccharide component of conjugate vaccines may be subject to gradual hydrolysis at a rate that may vary with the type of conjugate, the type of formulation or adjuvant, the type of excipients and conditions of storage. The hydrolysis may result in reduced molecular size of the pneumococcal polysaccharide component, in a reduction in the amount of the polysaccharide bound to the protein carrier and in a reduced molecular size of the conjugate.

The structural stability of the oligosaccharide chains and of the protein carrier vary between different conjugate vaccines.

Tests should be conducted before licensing to determine the extent to which the stability of the product has been maintained throughout the proposed validity period. The vaccine should meet the specifications for final product up to the expiry date.

Molecular sizing of the final product may not be feasible. However, to ensure that the integrity of the conjugate is preserved, molecular sizing may be carried out at an intermediate level, before formulation of the multivalent vaccine. The antigen content of each serotype conjugate may be determined by a quantitative serological assay.
The desorption of antigen from aluminium-based adjuvants, if used, may take place over time. The level of adsorption should be shown to be within limits agreed by the NRA, unless data are available to show that the immunogenicity of the final product is not dependent upon adsorption of the antigen to the adjuvant.

Accelerated stability studies may provide additional supporting evidence of the stability of the product but cannot replace real-time studies.

When any changes are made in the production procedure that may affect the stability of the product, the vaccine produced by the new method should be shown to be stable.

The statements concerning storage temperature and expiry date appearing on the label should be based on experimental evidence, which should be submitted for approval to the NRA.

A.8.2 Storage conditions
Storage conditions should be based on stability studies and approved by the NRA.

Storage of both liquid and freeze-dried vaccines at a temperature of 2–8 °C has been found to be satisfactory. The stability of pneumococcal conjugate components varies with serotype of the capsular polysaccharide.

A.8.3 Expiry date
The expiry date should be approved by the NRA and based on the stability of the final product as well as the results of the stability tests referred to in section A.8.1.

Part B. Nonclinical evaluation of new pneumococcal conjugate vaccines
Details on the design, conduct, analysis and evaluation of nonclinical studies are available in WHO guidelines on nonclinical evaluation of vaccines (47).

Nonclinical testing is a prerequisite for the initiation of clinical studies in humans and includes immunogenicity studies (proof of concept) and safety testing in animals. The vaccine lots used in nonclinical studies should be adequately representative of the formulation intended for clinical investigation and, ideally, should be the same lots used in clinical studies. If this is not feasible, the lots used clinically should be comparable to those used in the nonclinical studies in terms of potency, stability and other characteristics of quality.

With specific regard to pneumococcal conjugate vaccines, studies in animals would be expected to provide data on immune responses to the vaccine as part of the routine assessment of toxicokinetics. No single species can be recommended for these studies but manufacturers may find it useful to look at the data that have been generated for licensed pneumococcal conjugate vaccines.
that are in the public domain. It is important to appreciate that these data do not reliably predict a dose or range of doses of antigens that might be appropriate for study in humans, but such studies should demonstrate that a new pneumococcal vaccine elicits boostable immune responses in animals.

Part C. Clinical evaluation of pneumococcal conjugate vaccines

C.1 Considerations for clinical studies
This section addresses some issues that are specific to, or particularly relevant for, the clinical development of pneumococcal conjugate vaccines. The recommendations made should be considered in conjunction with the general principles described in Guidelines on clinical evaluation of vaccines: regulatory expectations (48) and should be viewed in the light of data on the safety, immunogenicity and effectiveness of pneumococcal conjugate vaccines that may become available in the future.

The section does not make any recommendations for the selection of serotypes to be included in a new pneumococcal conjugate vaccine. The selection process should take into consideration the relative frequencies of serotypes that cause IPD in the target population in different geographical regions.

Section C.2 considers the content of the clinical development programme applicable to pneumococcal conjugate vaccines intended primarily for the prevention of IPD and for administration to infants and toddlers. For reasons explained in General considerations, the potential efficacy of new pneumococcal conjugate vaccines for preventing IPD in this age group will be assessed based on studies of immune responses. Specific consideration is given to the immune response parameters of interest, the selection of licensed comparator vaccines, comparisons of immune responses to serotypes included in a new vaccine and in licensed comparator(s), and evaluation of immune responses to serotypes that are included only in a new vaccine.

Section C.3 briefly considers the clinical assessment of the potential for pneumococcal conjugate vaccines to prevent IPD in older children and adults (including the elderly) and to prevent non-invasive pneumococcal infections (e.g. pneumonia or otitis media).

Section C.4 considers the data on safety and effectiveness that should be collected following first approval of a new pneumococcal conjugate vaccine.

C.2 Assessment of immune responses
C.2.1 Assays to assess serotype-specific antibody responses
Immune responses to pneumococcal conjugate vaccines can be assessed by:
Determination of serotype-specific IgG antibody geometric mean concentrations (GMCs) based on measurement of binding to polysaccharides (e.g. using an ELISA method). Appendix 1 provides a detailed consideration of the development and standardization of ELISA methods, including:

- alternative methods to ELISA for measurement of serotype-specific IgG concentrations;
- the need to use a reference standard and quality control (QC) sera for IgG assays;
- the need to bridge new assays (whether ELISA or not) to the WHO reference assay and the option of deriving alternative threshold values when using new assays that correspond to 0.35 µg/ml based on a well-justified rationale.

Determination of serotype-specific functional antibody titres using an OPA (49). The conduct of OPAs is addressed in Appendix 1.

When comparing immune responses to pneumococcal conjugate vaccines following completion of the infant immunization series, it is recommended that the primary analysis should be based on IgG concentrations (see C.2.2.1). Secondary analyses should include a comparison of OPA titres (see C.2.2.2). The assessment of immune responses to booster doses is discussed in section C.2.3.

C.2.2 Evaluation of immune responses following the primary series

C.2.2.1 Selection of licensed comparator(s)

As long as the 7vPnC vaccine that has been evaluated in clinical studies of protective efficacy remains on the market it is recommended that the immune responses to this vaccine and to a new pneumococcal conjugate vaccine should be directly compared in prospective randomized studies in infants. Such studies provide the basis for bridging the protective efficacy conferred by the 7vPnC vaccine against IPD that was demonstrated in randomized controlled studies and in post-licensure studies of effectiveness to the new vaccine on the basis of comparable serotype-specific immune responses.

It is expected that the 7vPnC vaccine will become unavailable at some time in the future. Comparisons of immune responses should therefore be made between a new vaccine and at least one licensed vaccine for which immune responses were directly compared with the 7vPnC vaccine during the clinical development programme. Thus, licensure of a new pneumococcal conjugate vaccine would be based on a “bridge to a bridge” back to the data on efficacy and effectiveness for the seven serotypes in the 7vPnC vaccine.
Selection of the licensed pneumococcal conjugate vaccine(s) to be used as the comparator(s) will require very careful justification and must be discussed with NRAs. It is recommended that preference be given to selecting licensed comparators for which some effectiveness data are already available that lend support to the immunogenicity data on which their approval was based, together with a substantial safety database. Consideration should also be given to choosing licensed comparator(s) that have the highest number of serotypes in common with the new vaccine.

NRAs may be reluctant to approve a new pneumococcal conjugate vaccine on the basis of comparison with vaccines that are not actually licensed in their countries. However, once several pneumococcal conjugate vaccines have been approved in various countries, it may not be feasible for a new vaccine to be compared with every licensed vaccine. It is recommended that NRAs consider the acceptability of the licensed comparators used in clinical studies based on all the data available in the public domain regarding their safety, immunogenicity and effectiveness.

Whatever the licensed comparator(s) selected for clinical studies, comparisons of immune responses should follow the guidance provided in the following sections. Assessments of immune responses to serotypes that are and are not common to the new vaccine and the licensed comparator(s) require different approaches as described in C.2.2.3.

C.2.2.2 Schedules and populations

Immune responses to pneumococcal conjugate vaccines vary according to the schedule used, the population studied, and the antigen composition and nature of the vaccines that are administered concomitantly. It is not feasible to study new vaccines with every possible schedule in current use or in a very large range of geographical regions, nor is it possible to evaluate the effects of concomitant administration with a large range of vaccines in routine use (see section C.2.5). Manufacturers should justify the relevance of the clinical data provided to each country in which approval is sought and should discuss the basis for extrapolation of the findings.

For example, immune responses following a 2, 3 and 4 months schedule within a specific population are usually lower than response following a more relaxed 3-dose schedule (e.g. 2, 4 and 6 months). Documentation of satisfactory immune responses with the former schedule therefore supports the expectation that satisfactory immune responses would also be observed with the latter schedule. However, the local and systemic reactogenicity associated with a vaccine may also differ between schedules within a specific population, so that there is still a need to collect some safety data with other schedules proposed for approval (e.g. 2, 4 and 6 months).
Manufacturers may also choose to investigate immune responses after two doses in infancy (such as dosing at 2 and 4 months or 3 and 5 months). An exploration of immune responses after two or three doses in infants is to be encouraged since it is possible that, for certain vaccines administered according to specific schedules, there is no advantage in a third dose. The importance of assessing immune responses to additional doses after completion of any infant immunization series is addressed in section C.3.

C.2.2.3  Primary analysis

In the following sections the references to percentages reaching IgG concentrations ≥0.35 µg/ml are based on the WHO reference ELISA, as explained in General considerations and in Appendix 1. It is recognized in section C.2.1 and in Appendix 1 that it may be acceptable for manufacturers to employ an alternative and well-justified threshold value when using a specific in-house assay. Any alternative threshold value that is proposed should be shown to correspond to 0.35 µg/ml in a well-conducted bridging assay against the WHO reference ELISA. If the justification for using an alternative threshold value is considered to be acceptable, it would be used wherever the text that follows mentions 0.35 µg/ml.

The primary analysis should be based on IgG concentrations measured approximately 4 weeks after completion of the primary infant immunization series; IgG responses to serotypes shared between a new vaccine and the licensed comparator and to serotypes found only in a new vaccine should be regarded as co-primary but the analyses require different approaches as described below.

The predefined margins of non-inferiority for each end point should be justified (50, 51) and the effects of multiplicity should be taken into consideration in the statistical analysis plan. It is essential that the sample size is large enough to provide adequate power for the planned analyses; however, manufacturers may be able to provide justification for basing the calculation of sample size on a specific parameter if the total study size would otherwise become unmanageable. Such proposals need to be reviewed on a case-by-case basis.

There should at least be a measurable immune response to each serotype included in the new vaccine. Protocols should propose a definition for a measurable response that takes into account the performance characteristics of the assay.

For the serotypes common to the new vaccine and the licensed comparator

The end-points used in the primary analysis should be:

- the percentage of subjects with IgG ≥0.35 µg/ml, and
- the serotype-specific IgG GMC ratios.

It may be that the IgG responses to one or more serotypes meet the predefined non-inferiority criteria applied to percentages reaching the threshold
value but do not meet the predefined non-inferiority criteria applied to the comparison of GMCs – or vice versa. In this situation, meeting one of the two sets of criteria should be considered adequate for approval. If IgG responses for one or more serotypes fail to meet both sets of criteria, the NRA should take into consideration the disease burden associated with the serotype(s) when considering whether or not to approve the vaccine. In addition, if effectiveness data are already available for use of the new vaccine in other countries or regions, these may be used to assist the decision-making process. It may also be helpful to take into account the secondary immunogenicity analyses.

For serotypes found only in the new vaccine

Based on the serotype-specific demonstration of efficacy and effectiveness of the 7vPnC vaccine, there is a reasonable rationale for comparing proportions that achieve \( \geq 0.35 \mu g/ml \) against each serotype contained only in the new vaccine with any serotype in the licensed comparator that achieves the lowest percentage \( \geq 0.35 \mu g/ml \).

In the event of failure to elicit an IgG response to one or more serotypes that is at least comparable with the lowest response to any of the serotypes common to both vaccines, the issues mentioned above with regard to disease burden and any existing effectiveness data would again need to be taken into account.

If the NRA considers that, in the situations described above, it would still be appropriate to approve the new vaccine it is recommended that:

- The prescribing information makes clear the possible limitations of vaccine efficacy.
- Attention should be paid to the feasibility of estimating vaccine effectiveness in the post-approval period for the specific serotype(s) for which the predefined criteria were not met. The post-approval data may be used to indicate that the immune responses to the serotype(s) are sufficient to confer some protection against IPD. The feasibility and speed with which data could be generated will depend on the frequency of IPD associated with the serotype(s) in question. The generation of effectiveness data is considered in section C.2.4 below.

C.2.4 Secondary analyses

IgG concentrations

Since there is no definitive serotype-specific immunological correlate of protection established for pneumococcal conjugate vaccines, it is most important that the primary analysis of immune responses following completion of the infant immunization series is accompanied by other comparisons, including RCD plots. For any serotype that is common to the vaccines that have been compared, the RCDs
should be carefully scrutinized for any divergence of the curves. If divergence is observed, it is recommended that attention be given to the feasibility of generating serotype-specific vaccine effectiveness data, as mentioned above and in section C.2.4. RCD plots should also be generated for serotypes found only in the new vaccine but the review of these data should be seen as exploratory.

**OPA data**

The functional antibody responses (based on OPA assay data) to individual serotypes should be determined in a randomized subset of vaccinated subjects within some or all of the clinical studies. The OPA assay used by an individual manufacturer should be well validated. Issues surrounding the conduct of OPA assays are considered in Appendix 1.

At present, the interpretation of OPA data is made difficult by the fact that, while reaching a titre ≥1:8 indicates the presence of functional antibody, a titre that might correlate with protection against IPD due to any one serotype is unknown. For this reason it is recommended that comparisons of OPA titres that are common to the new vaccine and the licensed comparator focus on serotype-specific geometric mean titre (GMT) ratios. In addition, the serotype-specific RCD plots should be compared. OPA GMTs and RCD plots should also be generated for serotypes found only in the new vaccine but the review of these data should be seen as exploratory.

**C.2.2.5 Other possible analyses**

Manufacturers may choose to evaluate other parameters that are of interest but would not currently be seen as essential for study and inclusion in the application dossier. These include:

- antibody avidity; and
- effects on nasopharyngeal carriage, which may be assessed before and/or after initial approval.

**C.2.3 Post-primary series (booster) doses**

**C.2.3.1 Immune memory**

The clinical development programme should generate data to demonstrate that a new pneumococcal conjugate vaccine induces an immune memory response during the infant immunization series. These data can be obtained as part of the assessment of immune responses to booster doses of the new vaccine (see below). Administration of a non-conjugated pneumococcal vaccine (e.g. 23-valent polysaccharide vaccine) to children aged less than 2 years, who received conjugated vaccine in infancy, for the purpose of assessing prior induction of immune memory is not recommended. There are concerns that this practice may
result in immune hyporesponsiveness on further encounters with pneumococcal polysaccharides (i.e., on natural exposure or on receipt of further doses of a pneumococcal conjugate vaccine).

C.2.3.2 Rationale for assessing responses to post-primary series (booster) doses

The effectiveness data currently available from the routine use of the 7vPnC vaccine in developed countries are based on administration of 2 or 3 doses during infancy and a booster dose in the second year of life (from 11 months onwards). Experience gained with other polysaccharide conjugate vaccines has indicated the importance of immunological memory, adequate circulating antibody, and indirect (herd) protection to provide protection against invasive disease. Although clinical trials in developing countries have demonstrated the efficacy of the experimental 9vPnC vaccine over approximately 3–6 years following administration to infants on the EPI schedule without a subsequent dose in the second year of life (52) it remains to be seen whether this immunization strategy will provide long-term protection against IPD comparable with that achieved by regimens that employ a post-primary series booster dose. In addition, children at particular risk of IPD and/or with immunodeficiency probably need a post-primary series booster dose (53).

Clinical development programmes for new pneumococcal conjugate vaccines should therefore include studies in which immune responses to booster doses are measured and compared with responses to a licensed comparator(s) in a predefined secondary analysis. However, the optimal timing of the booster dose is unknown and probably varies according to the schedule and the concomitant vaccines in the infant immunization series. In most cases, booster doses are given at least 6 months after the last dose of the primary series and between the ages of 12 and 24 months but in some settings there may be reasons to boost earlier (e.g., at around 9 months). Ideally, clinical studies should investigate administration of booster doses at various times, although it must be recognized that it is not feasible to examine all possible permutations. Some of these data may be generated after initial approval of a new vaccine.

It is recommended that subsets of subjects be identified for longer-term follow-up of persistence of immunity after administration of booster doses. These data may be provided after first approval. Waning of antibody concentrations over time is inevitable and should not be interpreted per se to indicate the need for a booster dose. It is important that longer-term antibody concentrations be viewed in conjunction with effectiveness data to assess the potential need for additional doses later in life to maintain protection.

C.2.3.3 Comparisons of immune responses to booster doses

The evaluation of immune responses to booster doses should be based primarily on comparisons of immune responses at approximately 4 weeks post-booster dose
between groups of children who received the same pneumococcal conjugate vaccine (i.e. either the new vaccine or the licensed comparator) for the primary series and for boosting. Induction of immune memory during infancy should be associated with higher post-boost antibody concentrations in subjects who received a primary series in infancy than in age-matched unvaccinated children. If there is already routine use of licensed pneumococcal conjugate vaccine(s) in infants at study sites, it will not be impossible to compare responses to a single dose in the second year of life between previously vaccinated and unvaccinated groups for serotypes that are common to both vaccines. However, an assessment of booster responses to any additional serotypes in the new vaccine could be made by administering it to a subset of children who received the licensed comparator in infancy.

Measurement of pre- as well as post-boost antibody concentrations necessitates collection of an extra blood sample and is not considered to be necessary in all studies. However, it is preferred that at least some information on pre-boost antibody concentrations and/or titres should be generated during the clinical development programme. One possible way to do this is to randomly assign subjects to provide either a pre-boost or a post-boost blood sample at the time of initial randomization to vaccine group. These data allow changes in antibody levels from post-primary series to pre-booster to be assessed. In most studies, post-boost blood samples are obtained 4 weeks after the dose. The increase in antibody levels would be expected to start very early in those who are already primed. Some exploration of immune responses at less than 4 weeks post-booster dose in randomized subsets could be informative.

Immune responses to booster doses of pneumococcal conjugate vaccines would be expected to be very high for each of the serotypes included in the vaccine given in the infant immunization series. For this reason, comparisons between vaccine groups based on percentages reaching serotype-specific IgG concentrations ≥0.35 µg/ml or OPA titres ≥1:8 (or other relatively low cut-off values) would not be helpful because they would not adequately detect any differences between vaccine groups. It is therefore recommended that the comparisons of responses to booster doses focus on the ratio of the post-booster value to the post-primary value for the IgG GMCs or the OPA GMTs. It is also important that RCD plots are provided and inspected for any divergence in the curves.

C.2.4 Immune responses to carrier proteins

To date, the carrier proteins used in licensed pneumococcal conjugate vaccines have included a non-toxic diphtheria toxin molecule (CRM197), diphtheria toxoid, tetanus toxoid and Protein D from Haemophilus influenzae.

Administration of pneumococcal conjugate vaccines that employ diphtheria or tetanus toxoid or CRM197 as carrier(s) has been found to enhance the relevant anti-toxin antibody levels, but not to a sufficient extent to replace
routine immunization with diphtheria or tetanus toxoid-containing vaccines. Co-administration of a new pneumococcal conjugate vaccine with routine infant and toddler vaccines (i.e. containing diphtheria and tetanus toxoids) could result in high anti-toxin levels. Careful attention should be paid to the reactogenicity observed in these circumstances of use since increased rates of some reactions could be associated with high anti-toxin levels. As discussed in section C.2.5, data should be generated on anti-toxin levels on co-administration of a new pneumococcal conjugate vaccine with representative licensed vaccines in routine use.

It is possible that a carrier protein itself might elicit an immune response that confers some protection against an infectious disease. If a manufacturer wished to pursue such a claim, an appropriate clinical development programme would need to be discussed with NRAs.

C.2.5 Concomitant administration with other vaccines

Accumulation of data on the safety and immunogenicity of new pneumococcal conjugate vaccines when co-administered with other infant and toddler vaccines is essential. Concomitant administration of polysaccharide conjugates with other vaccines in routine use, which can include other conjugated vaccines, may give rise to lower immune responses to one or more of the co-administered antigens (i.e. immune interference), although the clinical significance of the observed phenomena is not always clear. The data on the effects of co-administration that are available at the time of initial licensure may be expanded in post-approval studies. It is sufficient that only some of the clinical studies include a formal assessment of the effects of co-administration on immune responses.

Studies of the effects of co-administration should include vaccines that are representative of types that, for reasons of convenience and compliance, are very likely to be given at the same clinic visits during routine use of a new pneumococcal conjugate vaccine. Because of the very large range of licensed vaccines that may need to be co-administered with pneumococcal conjugate vaccines in infants and toddlers, using a variety of schedules, it is not feasible for manufacturers to study every possible permutation. Immune responses to the conjugated pneumococcal serotypes and to the co-administered antigens should be evaluated. Limitations of sera volumes commonly make it necessary to perform an additional randomization step to select sera to be used in the different antibody assays.

The range and design of studies should take into account the following general statements regarding schedules and co-administered vaccines:

- If there is no potentially clinically significant effect on immune responses observed on concomitant administration using an early infant schedule (e.g. 6, 10 and 14 weeks, or 2, 3 and 4 months), it is unlikely there any such effect would be observed on co-administration using more relaxed schedules (such as 2, 4 and 6 months) in a similar
population, since the magnitude of immune responses is generally higher in the latter case. In contrast, an extrapolation of no effect observed with co-administration on a relaxed schedule to use on an early infant schedule in a similar population is not possible.

- If no potentially clinically significant effects on immune responses are observed on concomitant administration of a new pneumococcal vaccine with a complex vaccine (e.g. a hexavalent vaccine containing DTaP, IPV, HBV and Hib), with or without a meningococcal conjugate vaccine, it is reasonable to extrapolate the findings to co-administration with less complex vaccines (i.e. containing a lower total number of antigens, such as DTaP–IPV–Hib) – but not vice versa.

The most straightforward way to assess the effect of co-administration on immune responses is by means of random selection of sera obtained from subjects who have received a new or a licensed pneumococcal vaccine along with exactly the same routine vaccine(s) on the same schedule within the same study. This approach assumes that the licensed comparators used in these studies have already been approved for co-administration with the types of infant or toddler vaccines that have been selected for study. Thus, it also assumes that any immune interference that may have been observed in studies with the licensed comparator(s) was not considered to be potentially clinically significant.

The primary objective of these studies would be the same as in all other studies that directly compare immune responses between a new pneumococcal conjugate vaccine and licensed comparator(s); the focus would therefore be on the responses to the pneumococcal serotypes as already described.

Comparisons of immune responses to all other co-administered antigens should be listed among the preplanned secondary analyses. If the results indicate that immune responses are lower to one or more of the antigens on co-administration with a new pneumococcal conjugate vaccine than with the licensed vaccine(s), NRAs will need to consider the potential clinical consequences on a case-by-case basis. Consideration should be given to prior data on co-administration of each antigen with the 7vPnC or other licensed comparator. There may be a greater concern if the selected licensed comparator had itself depressed the immune response to an antigen compared with the 7vPnC vaccine.

Any increase in adverse reactions that is observed on co-administration will need to be weighed against the convenience of administering multiple vaccines during a single health-care contact.

C.2.6 **Studies in special populations**

Certain underlying conditions (e.g. immunodeficiency and asplenia) predispose to pneumococcal infections. Some, but not all, populations with these conditions may also mount lower than usual immune responses to
pneumococcal conjugate vaccines. In populations with a high prevalence of conditions predisposing to IPD, clinical studies may be conducted specifically to assess the safety and immunogenicity of new pneumococcal vaccines. These studies may be performed before or after initial licensure and should include an assessment of OPA titres in a subset of sera.

C.3 Other possible indications for use

On the basis of safety and immunogenicity studies alone, there is currently no rationale for approving new pneumococcal conjugate vaccines for prevention of IPD following administration to subjects older than 2 years or for prevention of pneumonia or otitis media. This is because no immunological correlate of protection has been identified and the available data do not support a recommendation for any threshold value that might be used as a benchmark. The clinical development programmes to support these indications require different approaches but no definitive guidance can be given regarding the clinical studies that should be performed.

Pneumococcal conjugate vaccines have been approved for the prevention of otitis media and pneumonia caused by *S. pneumoniae*. Thus far, approval of pneumococcal conjugate vaccines for the prevention of these indications has been based on efficacy and effectiveness data.

No pneumococcal conjugate vaccine is yet approved for prevention of pneumococcal disease in the elderly but an efficacy study was in process at the time of preparation of this annex. It is possible that efficacy and immunogenicity data obtained during this study may eventually allow for approvals based on a comparison of safety and immunogenicity data only.

C.4 Post-marketing studies of safety and effectiveness

The manufacturer has a responsibility to assess safety and effectiveness following initial approval of a new pneumococcal vaccine. NRAs should ensure that adequate plans are in place regarding these activities at the time of first licensure of a new pneumococcal conjugate vaccine. Basic principles for conducting post-licensure studies and surveillance are outlined in the Guidelines on clinical evaluation of vaccines: regulatory expectations (48). Specific commitments should be 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. Pre- and post-approval discussions between vaccine manufacturers responsible for placing the product on the market and national and international public
health bodies are therefore essential for ensuring that reliable effectiveness data are collected in the post-marketing period in selected countries/regions. Robust estimates of effectiveness can be obtained only in geographical locations in which a new pneumococcal conjugate vaccine has been introduced into routine immunization programmes and where suitable infrastructure is in place to identify cases of IPD. Publications produced by the WHO Expanded Programme on Immunization are important sources of information to assist in the monitoring of vaccine effectiveness once new vaccines are introduced into immunization programmes. A manual outlining approaches to monitoring the impact of S. pneumoniae conjugate vaccination on pneumococcal disease burden was being developed.

At present it is not known whether subjects who have completed an infant immunization series and received a subsequent booster dose will require further booster doses to maintain long-term protection against all serotypes in the vaccine. The need for further doses may depend on several factors such as waning antibody levels, the lack of natural boosting associated with low rates of circulation of some or all serotypes in the vaccine, the numbers of cases identified from disease surveillance, and estimates of herd immunity that result from routine use. It is also important to assess the effects of widespread vaccination on the incidence of IPD caused by non-vaccine serotypes to evaluate any beneficial effects and/or evidence of serotype replacement following vaccine introduction. The duration of monitoring of effectiveness will need to be reviewed continually since it should be driven by the findings.

Part D. Recommendations for national regulatory authorities

D.1 General
The general recommendations for control laboratories contained in Guidelines for national authorities on quality assurance for biological products (54) should be applied.

D.2 Release and certification
A vaccine lot should be released only if it fulfils national requirements and/or Part A of these Recommendations. A statement signed by the appropriate official of the NRA should be provided at the request of the manufacturing establishments and should certify that the lot of vaccine in question satisfies all national requirements as well as Part A of these Recommendations. The release certificate should state the number under which the lot was released by the NRA and the number appearing on the labels of the containers. Importers of
pneumococcal conjugate vaccines should be given a copy of the official national release document. The purpose of the certificates is to facilitate the exchange of vaccines between countries. (See appendices 2–4 for the summary protocol for manufacturing and control of pneumococcal conjugate vaccine; certification by the manufacturer and a model certificate for the release of pneumococcal conjugate vaccines.)

Given the lack of a suitable animal model that will predict the potency of all pneumococcal serotypes, the strategy for the control of the vaccine is dominated by the use of tests for physicochemical characterization and purity. These tests focus on criteria to ensure each vaccine lot is consistent with the specification of the vaccine lots used in the definitive clinical trials that confirmed their safety and immunogenicity.

D.3 **Consistency of manufacture**

The NRA should satisfy itself that adequate control of the manufacturing, shipping and storage of the pneumococcal conjugate vaccine has been achieved. NRAs may consider that a formal clinical lot-to-lot consistency study is not necessary if adequate and satisfactory data are provided to support consistency of manufacture. However, several different lots of the product should be used in randomized studies and should elicit comparable immune responses in similar populations.

Pneumococcal conjugate vaccines are manufactured from purified components by a clearly defined validated chemical process. Any changes in production or formulation of the vaccine should be reported to the NRA and a decision regarding the potential need for additional clinical data should be made on a case-by-case basis. The decision should take into account the likelihood of such changes affecting the quality, consistency, structural integrity and immunogenicity of the vaccine and should consider the possible cumulative effect of multiple modifications that individually may be regarded as minor.

**Authors**

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References


Appendix 1

Methodological considerations: quantification of IgG antibodies for type-specific pneumococcal capsular polysaccharide in human sera

Introduction
This appendix provides guidance on the standardization and validation of methods for measurement of pneumococcal serotype-specific IgG antibody concentrations and functional antibody titres.

Measurement of serotype-specific IgG antibody
Serotype-specific IgG antibody should be the primary parameter used to compare the immune responses to new and licensed pneumococcal conjugate vaccines.

Assay development
The ELISA that was used to evaluate serotype-specific IgG concentrations in sera obtained from subjects enrolled into the three protective efficacy trials with the 7vPnC or experimental 9vPnC vaccines included a pre-adsorption step with pneumococcal C-polysaccharide (C-PS) to reduce the content of non-serotype-specific antibody. On the basis of data from the three studies using the original ELISA method, a threshold IgG antibody concentration of ≥0.35 µg/ml was recommended for use as a benchmark when comparing immune responses between vaccines (1).

It was subsequently shown that the concentration of non-serotype-specific antibody in adult sera can be reduced further by pre-adsorption with both C-PS and 22F polysaccharide. This double pre-adsorption approach is recommended because it reduces the potential for over-estimation of serotype-specific IgG antibody concentrations and improves the correlation between results of the ELISA and OPA titres (2, 3). This ELISA was established as the WHO reference assay; the detailed protocol for the method is available elsewhere (4).

The original ELISA method that was used to establish the threshold value of ≥0.35 µg/ml differed thus from the WHO reference ELISA in that it did not include a 22F pre-adsorption step. It was later shown that, using the original ELISA protocol, pre-adsorption of sera with C-PS and 22F had a minimal effect on estimations of IgG concentrations in a selection of sera from vaccinated infants compared with the original assay that used only C-PS pre-adsorption (5).

Despite the establishment and widespread recognition of the WHO reference ELISA, several laboratories have developed their own in-house ELISA
methods that include modifications of the original assay protocol. Experience gained with various in-house methods has demonstrated that relatively small changes in assay methodology, such as the source of polysaccharides used to coat the wells, can affect assay performance. In addition to these modified ELISA methods, new assays that measure serotype-specific IgG concentrations have been developed. For example, multiplex antibody binding assays can be used to determine multiple serotype-specific IgG concentrations simultaneously and so reduce the volumes of sera required from individual subjects. All in-house assays used in immunogenicity studies designed to evaluate protection against IPD need to be bridged to the WHO reference assay in order to maintain the link between immune responses to vaccination and the demonstration of protective efficacy against IPD conferred by the seven conjugated polysaccharides in the 7vPnC vaccine.

Reference laboratories

Two WHO reference laboratories have been established to facilitate the standardization of ELISA methods. These are located at the Institute of Child Health, London, England, and at the Bacterial Respiratory Pathogen Reference Laboratory, University of Birmingham, Birmingham, AL, USA.

Reagents

A reference serum (89-SF; Center for Biologics Evaluation and Research) and a quality control panel of sera (National Institute for Biological Standards and Control) have been established using blood samples collected from adults vaccinated with 23-valent polysaccharide vaccine. While the applicability of an adult reference serum when determining IgG concentrations in sera obtained from vaccinated infants has been debated, it was not considered feasible to produce an infant reference serum pool.

The 89-SF serum has assigned serotype-specific IgG concentrations that were developed using a single C-PS adsorption step (6). Addition of a 22F pre-adsorption step would be expected to reduce serotype-specific IgG concentrations and is therefore not recommended. Indeed, it has been shown that pre-adsorption with both C-PS and 22F resulted in inflation of the GMC values for some serotypes by more than 25%. Because of the depletion of supplies of 89-SF, a replacement reference serum is being developed and will be bridged to 89-SF. The new reference (ref 007sp) will be calibrated against the 89-SF after adsorption with both C-PS and 22F polysaccharides.

The assigned IgG concentrations in the QC panel of sera were based on pre-adsorption with C-PS and 22F; the sera should therefore be subjected to double pre-adsorption before use. It is expected that a new QC panel of sera will be established to assist in the standardization of new assays and to monitor assay performance.
Assay validation

In the clinical development programme for each new pneumococcal conjugate vaccine, it is essential that validated assays be conducted in centralized laboratories. Assay validation involves demonstrating that the performance characteristics of the method meet the requirements for the intended use of the method. The protocols for assay validation studies should identify, and justify the choice of, parameters to be studied, and include the predefined acceptance criteria. There should be a detailed description of processing and storage of samples, reference standards and reagents, and generation of the calibration curve.

Extensive general guidance is available regarding assay validation and is also applicable to ELISA methods for estimating pneumococcal serotype-specific IgG concentrations; detailed guidance is therefore not given here. However, validation studies should adequately describe the following attributes of the assay:

- specificity,
- accuracy,
- precision (including repeatability, intermediate precision and reproducibility),
- detection limit,
- quantitation limit,
- linearity,
- range,

and robustness should be documented during assay development.

Assay standardization and bridging to the original ELISA

Inter-laboratory assay variation can be attributed to the laboratory protocol (i.e. the reagents, the reference standards, and the conditions and times for protocol steps) and the data reduction method used (i.e. non-parallelism between standard and serum dilution curves, the functions used to model standard curves, and the calculation protocols).

In-house methods for measurement of serotype-specific IgG concentrations should be evaluated using a performance-based approach that allows laboratories to optimize certain assay parameters and reduce rates of systematic errors. One approach to evaluating assay performance is to determine IgG concentrations for the reference and QC panel sera using the in-house method and compare these values with the assigned concentrations (7). In this way, the data can be used to estimate the level of agreement between the in-house assay and the WHO reference ELISA. That is, if the results are within predefined and justified acceptance criteria, it can be expected that the in-house assay will
generate results from unknown sera that are comparable with those obtained using the WHO reference assay.

A statistical approach has been proposed for comparisons of performance between in-house and WHO reference assays (7). Alternative statistical methods that may be used to determine the agreement between laboratories or between one assay and the QC panel sera include Lin’s concordance correlation coefficient and other regression procedures (e.g. a Deming regression). It is recommended that laboratories should obtain expert statistical advice when undertaking these comparisons.

As explained above, in-house assays used to evaluate immune responses to pneumococcal conjugate vaccines intended for administration to infants and toddlers for the prevention of IPD need to be adequately and carefully bridged to the original ELISA protocol in order to maintain the link to the protective efficacy that has been demonstrated for the 7VPnC vaccine. Each in-house method that is to be used to evaluate protection against IPD by assessing serotype-specific IgG concentrations in sera obtained from vaccinated infants should be adequately bridged to the WHO ELISA.

The bridging process requires a study that is specifically designed to demonstrate comparable performance between the in-house assay and the WHO reference ELISA. Bridging studies should employ sera obtained from infants who have received the 7vPnC vaccine or, if this vaccine is no longer available, a suitable alternative licensed vaccine that contains at least the seven serotypes in the 7vPnC vaccine. The statistical approach to analysis of bridging study results is similar to that used for routine assay standardization.

Based on the performance of an in-house assay and the results of a bridging study, a laboratory may consider it appropriate to apply an alternative assay-specific threshold value when analysing the data as recommended in section C.2. The use of an alternative assay-specific threshold value would require a very detailed and robust justification of threshold equivalency, and it is recommended that this be discussed with NRAs before the clinical development programme reaches the stage of analysis of IgG concentration data.

**Determination of functional antibody using OPA**

An OPA provides a measurement of functional antibody, and it is recommended that functional antibodies be assessed in subsets of sera obtained in clinical studies with all new pneumococcal conjugate vaccines. However, there is no very well established threshold value that could be used to assist in interpretation of the data; the reasons for this include the lack of standardization of these complex assays, which are intrinsically variable because of the need to use reagents of a biological nature (e.g. cells, bacteria and complement) (8). A standardized and well-characterized assay, against which all other OPA methods can be bridged, is therefore needed.
A multi-laboratory study to assess comparability of OPA assays began in 2007. The five participating laboratories used the same assay protocol and method of data analysis that were in routine use at each site. Each laboratory received 24 samples and ran its OPA method for either 7 or 13 serotypes. All five laboratories used HL 60 effector cells that were differentiated using a similar protocol. Two laboratories used a multiplex platform and four used baby rabbit serum as the source of complement. There was no consistency among laboratories with regard to the pneumococcal isolates used in the assay.

Notwithstanding the diversity of the assays in use, the results obtained by the five laboratories showed a good level of agreement. There was particularly good agreement on the samples with negative titres, although the agreement on actual titres was poor.

It is clear that further standardization efforts are needed to permit comparison of OPA results from different laboratories and across clinical studies. Improvements in assay performance, such as the establishment of a reference serum, will provide more reliable results. Ultimately these efforts should also facilitate assessment of the correlation between OPA titres and protection. However, even using a single assay in one laboratory, the available data with the 7vPnC vaccine showed up to 10-fold differences in OPA GMTs between serotypes, suggesting that serotype-specific correlates will probably need to be derived.

**Correlation between IgG concentrations and functional antibody (OPA titres)**

Although it is recommended that the primary analysis of immune responses should be based on IgG concentrations, the serotype-specific functional antibody is regarded as the surrogate of protection. Thus, IgG concentrations determined by in-house assays should be assessed for correlation with OPA titres using sera from subjects who receive the new and reference pneumococcal conjugate vaccines in at least one clinical study.

**References**


Appendix 2

Summary protocol for manufacturing and control of pneumococcal conjugate vaccine

The following protocol, which is intended for guidance, indicates the information that should be provided as a minimum by the manufacturer to the national regulatory authority.

Information and tests may be added or deleted as required by the national regulatory authority. 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 for 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 and a copy of the leaflet that accompanies the vaccine container. If the protocol is being submitted in support of a request to permit importation, it must also be accompanied by a lot release certificate from the national regulatory authority of the country in which the vaccine was produced, stating that the product meets national requirements as well as the recommendations in Part A of this document.

Summary information on final lots

International nonproprietary name of product __________________________
Commercial name __________________________
Product licence (marketing authorization) number __________________________
Country __________________________
Name and address of manufacturer __________________________
Final packing lot number __________________________
Type of containers __________________________
Number of containers in this packing lot __________________________
Final container lot number __________________________
Number of filled containers in this final lot __________________________
Date of manufacture (filling) __________________________
Nature of final product (adsorbed) __________________________
Preservative and nominal concentration __________________________
Volume of each recommended single human dose __________________________
Number of doses per final container __________________________
Summary of the composition (include a summary of the qualitative and quantitative composition of the vaccine per human dose including the conjugate, any adjuvant used and other excipients):

Shelf-life approved (months) _____________________________

Expiry date _____________________________

Storage conditions _____________________________

The following sections are intended for the reporting of the results of the tests performed during the production of the vaccine, so that the complete document will provide evidence of consistency of production; thus, if any test has to be repeated, this must be indicated. Any abnormal results should be recorded on a separate sheet.

Detailed information on manufacture and control

Summary of starting materials

It is possible that a number of bulk lots are used to produce a single final lot. A summary of the bulk polysaccharide, activated polysaccharide, bulk carrier protein and bulk conjugate lots that contribute to the final lot should be provided.

Control of pneumococcal polysaccharides

Strain

Identity of *Streptococcus pneumoniae* strain used in vaccine _____________________________

Origin and short history _____________________________

Authority that approved the strain _____________________________

Date approved _____________________________

Master seed lot

Lot number _____________________________

Date working seed lot was established _____________________________

Working seed lot

Lot number _____________________________

Date working seed lot was established _____________________________

Control tests on working seed lot _____________________________

Culture media for the production of pneumococcal polysaccharides

Any components of animal origin _____________________________

Certificate for TSE-free _____________________________
Control of single harvests
List the single harvests and indicate the medium, dates of inoculation, temperature of incubation, dates of harvests, volumes, results of tests for bacterial purity and identity, the method and date of bacterial killing, the method of purification, and the yield of purified polysaccharide.

Control of purified polysaccharide
Lot number
Date of manufacture
Volume

Identity
Date of test
Method
Specification
Result

Moisture (for lyophilized intermediates)
Date of test
Method
Specification
Result

Polysaccharide content
Date of test
Method
Specification
Result

Protein impurity
Date of test
Method
Specification
Result

Nucleic acid impurity
Date of test
Method
Specification
Result
### Endotoxin content
- **Date of test**: 
- **Method**: 
- **Specification**: 
- **Result**: 

### O-acetyl content (for relevant polysaccharides)
- **Date of test**: 
- **Method**: 
- **Specification**: 
- **Result**: 

### Molecular size distribution
- **Date of test**: 
- **Method**: 
- **Specification**: 
- **Result**: 

### Control of modified polysaccharide (if applicable)
- **Lot number**: 
- **Method for activation**: 

### Extent of modification
- **Date of test**: 
- **Method**: 
- **Specification**: 
- **Result**: 

### Molecular size distribution
- **Date of test**: 
- **Method**: 
- **Specification**: 
- **Result**: 

### Control of carrier protein

#### Microorganisms used
- **Identity of strain used in carrier protein production**: 
- **Origin and short history**: 
- **Authority that approved the strain**: 
- **Date approved**: 

---

**Annex 3**

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Working seed lot
Lot number ____________________________
Date working seed lot was established ____________________________
Control tests on working seed lot ____________________________
Date of reconstitution of seed lot ____________________________

Culture media for production of carrier protein
Any components of animal origin ____________________________
Certificate for TSE-free ____________________________

Tests on carrier protein
Identity
Date of test ____________________________
Method ____________________________
Specification ____________________________
Result ____________________________

Purity
Date of test ____________________________
Method ____________________________
Specification ____________________________
Result ____________________________

Extent of derivatization (if applicable)
Date of test ____________________________
Method ____________________________
Specification ____________________________
Result ____________________________

Antigenic activity (for protein D derived from non-typable Haemophilus influenzae)
Date of test ____________________________
Method ____________________________
Specification ____________________________
Result ____________________________

Control of monovalent bulk conjugate
Production details of bulk conjugates
List the lot numbers of the individual polysaccharides and the carrier protein(s) used in the manufacture of the conjugate vaccine, the production procedure, date of manufacture and yield.
Tests on purified bulk conjugates

The tests listed below are usually performed on non-adsorbed conjugate bulks. Alternatively, they may be performed on adsorbed monovalent conjugate bulks, e.g. in case conjugate bulks are adsorbed to adjuvant individually before final formulation of the vaccine.

### Residual reagents (if applicable)

<table>
<thead>
<tr>
<th>Date of test</th>
<th>Method</th>
<th>Specification</th>
<th>Result</th>
</tr>
</thead>
</table>

### Pneumococcal polysaccharide content

<table>
<thead>
<tr>
<th>Date of test</th>
<th>Method</th>
<th>Specification</th>
<th>Result</th>
</tr>
</thead>
</table>

### Identity

<table>
<thead>
<tr>
<th>Date of test</th>
<th>Method</th>
<th>Specification</th>
<th>Result</th>
</tr>
</thead>
</table>

### Free polysaccharide content

<table>
<thead>
<tr>
<th>Date of test</th>
<th>Method</th>
<th>Specification</th>
<th>Result</th>
</tr>
</thead>
</table>

### Protein content

<table>
<thead>
<tr>
<th>Date of test</th>
<th>Method</th>
<th>Specification</th>
<th>Result</th>
</tr>
</thead>
</table>

### Free protein content (if applicable)

<table>
<thead>
<tr>
<th>Date of test</th>
<th>Method</th>
<th>Specification</th>
<th>Result</th>
</tr>
</thead>
</table>
### Ratio of polysaccharide to protein

<table>
<thead>
<tr>
<th>Date of test</th>
<th>Method</th>
<th>Specification</th>
<th>Result</th>
</tr>
</thead>
</table>

### Molecular size distribution

<table>
<thead>
<tr>
<th>Date of test</th>
<th>Method</th>
<th>Specification</th>
<th>Result</th>
</tr>
</thead>
</table>

### Sterility

<table>
<thead>
<tr>
<th>Method</th>
<th>Media</th>
<th>Volume tested</th>
<th>Date of inoculation</th>
<th>Date of start of test</th>
<th>Date of end of test</th>
<th>Specification</th>
<th>Result</th>
</tr>
</thead>
</table>

### Specific toxicity of carrier protein (if applicable)

<table>
<thead>
<tr>
<th>Method</th>
<th>Strain and type of animals</th>
<th>Number of animals</th>
<th>Route of injection</th>
<th>Volume of injection</th>
<th>Quantity of protein injected</th>
<th>Date of start of test</th>
<th>Date of end of test</th>
<th>Specification</th>
<th>Result</th>
</tr>
</thead>
</table>

Depending upon the conjugation chemistry used to produce the vaccine, suitable tests should also be included demonstrating that residual reagents and reaction by-products are below a specified level.

### Control of final bulk

*Lot number*
### Name and nature of adjuvant, if used
- Lot number
- Final concentration in the final bulk

### Name and nature of preservative, if used
- Lot number
- Final concentration in the final bulk

### Name and nature of stabilizer, if used
- Lot number
- Final concentration in the final bulk

### Test on final bulk
#### Sterility
- Method
- Media
- Volume tested
- Date of inoculation
- Date of end of test
- Specification
- Result

### Filling and containers
#### Lot number
- Date of sterile filtration
- Date of filling
- Volume of final bulk filled
- Filling volume per container
- Number of containers filled (gross)
- Date of lyophilization (if applicable)
- 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 product
#### Tests on final lot
##### Appearance
- Date of test
- Method
**Speciation**

<table>
<thead>
<tr>
<th>Specification</th>
<th>Result</th>
</tr>
</thead>
</table>

**Identity**

<table>
<thead>
<tr>
<th>Date of test</th>
<th>Method</th>
<th>Specification</th>
<th>Result</th>
</tr>
</thead>
</table>

**Sterility**

<table>
<thead>
<tr>
<th>Method</th>
<th>Media</th>
<th>No. of containers tested</th>
<th>Date of inoculation</th>
<th>Date of end of test</th>
<th>Specification</th>
<th>Result</th>
</tr>
</thead>
</table>

**Serotype-specific pneumococcal polysaccharide content**

<table>
<thead>
<tr>
<th>Date of test</th>
<th>Method</th>
<th>Specification</th>
<th>Result</th>
</tr>
</thead>
</table>

**Endotoxin content**

<table>
<thead>
<tr>
<th>Date of test</th>
<th>Method</th>
<th>Specification</th>
<th>Result</th>
</tr>
</thead>
</table>

**Total polysaccharide content**

<table>
<thead>
<tr>
<th>Date of test</th>
<th>Method</th>
<th>Specification</th>
<th>Result</th>
</tr>
</thead>
</table>

**Adjuvant content**

<table>
<thead>
<tr>
<th>Date of test</th>
<th>Nature and concentration of adjuvant per human dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method</td>
<td>Specification</td>
</tr>
<tr>
<td>--------</td>
<td>---------------</td>
</tr>
</tbody>
</table>

**Preservative content (if applicable)**

<table>
<thead>
<tr>
<th>Date of test</th>
<th>Method</th>
<th>Specification</th>
<th>Result</th>
</tr>
</thead>
</table>

**General safety test (if applicable)**

<table>
<thead>
<tr>
<th>Date of test</th>
<th>Method</th>
<th>Specification</th>
<th>Result</th>
</tr>
</thead>
</table>

**pH**

<table>
<thead>
<tr>
<th>Date of test</th>
<th>Method</th>
<th>Specification</th>
<th>Result</th>
</tr>
</thead>
</table>
Appendix 3

Certification by the manufacturer

Name of the manufacturer ________________________________

Certification by person from the control laboratory of the manufacturing company taking overall responsibility for the production and control of the vaccine.

I certify that Lot No. _______________ of Pneumococcal Conjugate Vaccine, whose number appears on the label of the final containers, meets national requirements and satisfies Part A of the WHO Recommendations to assure the quality, safety and efficacy of pneumococcal conjugate vaccines (WHO TRS 977).

Signature ____________________________________________
Name (typed) __________________________________________
Date ________________________________________________
Appendix 4

Model certificate for the release of pneumococcal conjugate vaccines

This certificate is to be provided by the national regulatory authority of the country where the vaccines have been manufactured, upon request by the manufacturer.

Certificate No. __________________________

LOT RELEASE CERTIFICATE

The following lot(s) of pneumococcal conjugate vaccine produced by ___________________________1 in ___________________________2, whose numbers appear on the labels of the final containers, meet all national requirements3 and Part A4 of the WHO Recommendations to assure the quality, safety and efficacy of pneumococcal conjugate vaccines (WHO TRS 977),5 and comply with Good manufacturing practices for pharmaceutical products6 and Good manufacturing practices for biological products7.

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 lot</th>
<th>Expiry date</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Director of the national regulatory authority (or other 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 4**

**Recommendations to assure the quality, safety and efficacy of influenza vaccines (human, live attenuated) for intranasal administration**

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Recommendations published by WHO are intended to be scientific and advisory in nature. The parts of each section printed in type of normal size have been written in such a form that, should a national regulatory authority so desire, they may be adopted as they stand as definitive national requirements or used as the basis for such requirements. The parts printed in smaller type are comments and recommendations intended as guidance for manufacturers and national regulatory authorities that may benefit from additional information. It is recommended that any modifications be made only on condition that they ensure that the vaccine is at least as safe and efficacious as that prepared in accordance with the recommendations set out below. In order to facilitate the international distribution of vaccine made in accordance with these recommendations, a summary protocol for the recording of results of tests is given in Appendix 1. The terms “national regulatory authority” and “national control laboratory” as used in these recommendations always refer to the country in which the vaccine is manufactured.
Introduction

The WHO requirements for influenza vaccine (live) date from 1979 (1). The purpose of these updated recommendations is to provide vaccine manufacturers and national regulatory authorities with applicable considerations and guidance in developing specific processes for assuring the quality, safety and efficacy of influenza vaccines (human, live attenuated) for intranasal administration, including their nonclinical and clinical evaluation.

These recommendations apply to influenza vaccines (human, live attenuated) for intranasal administration using embryonated hen’s eggs as substrates. It is expected that these vaccines will be produced using cell cultures as substrates and guidance is also provided for this eventuality. These recommendations are not specific for a particular form of influenza vaccine virus attenuation used to prepare the final influenza virus vaccine product. They should apply to the production and quality control of viruses intended for use in the manufacture of influenza vaccine (human, live attenuated) for intranasal administration, including reassortant viruses prepared either by classical reassortment methods or by reverse genetics techniques.

The recommendations (with possible modifications) are meant to apply to influenza vaccines (human, live attenuated) for intranasal administration, produced with seasonal vaccine strains for use during the inter-pandemic period as well as vaccines produced with strains for use during pandemics. However, these recommendations cannot anticipate every situation that may arise, and alternative considerations may be needed for specific public health circumstances. The first draft of this document was based on the requirements for influenza vaccines (live) from 1979 (1) and on the recommendations for the production and control of influenza vaccines (inactivated) (2). Sections on the preclinical, nonclinical and clinical evaluation were added to the updated recommendations to assure the quality, safety and efficacy of influenza vaccines (human, live attenuated) for intranasal administration. The section on testing animals for adventitious agents, which was included in the 1979 requirements, was removed from the updated recommendations as animal testing for that purpose is no longer generally recommended.

The recommendations in this document do not apply to the potential vector systems (i.e. other viral or bacterial hosts) that could be used to deliver the antigenic components of influenza viruses. Further recommendations may be developed in the future as additional strategies emerge for immunological control of influenza virus infections.

General considerations

Influenza is a significant cause of morbidity and mortality and each year has major social and economic impact throughout the world. Influenza viruses...
undergo continuous evolutionary change, which makes control of influenza challenging. In order to assist national regulatory authorities and manufacturers in control efforts, WHO provides annual reviews of epidemiological information and recommendations on the influenza viruses to use in vaccines in countries of the northern and southern hemispheres (3).

Many people require medical treatment and/or hospitalization, and excess mortality often accompanies viral influenza epidemics; the vast majority of those affected are elderly. Because the elderly constitute the most rapidly increasing sector of the population in many countries, the epidemiology of viral influenza can be expected to change accordingly, especially in high-income countries. Although mortality is typically highest among the elderly, influenza epidemics originate in settings that bring together immunologically susceptible individuals who are capable of spreading infection rapidly throughout a community, such as school-aged children. Infants are particularly susceptible to the severe consequences of viral influenza infections, but children of all ages may experience complications of influenza, including pneumonia and death.

At present, the most generally available means of influenza prophylaxis is vaccination. Potential means of prevention, other than personal and societal hygienic measures, include antiviral medications. However, the indefinite nature of exposure to influenza virus, which could necessitate protracted compliance with an antiviral medication regimen, as well as issues of potential or real emergence of drug-resistant virus strains, has prompted strategies to reserve chemoprophylaxis for specific circumstances (4, 5).

Inactivated influenza vaccines, which function mainly by inducing IgG antibodies specific for influenza virus haemagglutinins, have a long and solid record of use. The interest in live influenza virus vaccines stems from their potential to permit simplified administration by intranasal drops or spray (6). In addition, they stimulate not only systemic humoral immunity but also local and systemic immune protective mechanisms, including mucosal IgA antibodies and cellular immunity. The possibility of controlling influenza virus infection and illness by the use of live attenuated virus vaccines given by the intranasal route was thus extensively investigated during the latter half of the twentieth century. Live influenza virus vaccines arising from studies of “cold-adapted” donor strains have been used as an effective public health tool in industrialized countries including the Russian Federation (7) and the United States of America (8). Although current cold-adapted vaccines are manufactured in embryonated hen’s eggs, there is ongoing research to develop influenza vaccines (human, live attenuated) using other methods of virus attenuation and produced in cell culture.

The principle of a live vaccine for controlling a viral infection has a sound basis and has been used in preventing other viral infectious diseases, such as poliomyelitis, measles, mumps and rubella. Attenuated poliovirus vaccines are given orally to infect the cells of the intestinal tract; this stimulates protective
immune responses that mimic those occurring after natural poliovirus infection. By analogy, it may be possible to initiate a benign influenza virus infection in the nasopharynx with an attenuated influenza virus strain to give protection against the prevalent wild-type influenza strains.

The successful deployment of live attenuated virus donor strains depends on ensuring an appropriate balance between attenuation and immunogenicity. The aim is to produce an attenuated virus that incorporates the key immunizing antigens and antigenic determinants of circulating wild influenza viruses but retains the stable genetic and phenotypic characteristics of the attenuated donor strain when given to susceptible individuals on a wide scale. The ideal candidate would provide strong strain-specific protection and broad cross-reactivity, stimulate all categories of protective immunity, produce few or no symptoms in the most susceptible hosts, and be able to infect all hosts in whom specific protective immunity is lacking. Pragmatically, compromise on one or more of these features may be required.

The continuing commercial development and public health use of influenza vaccines made from live attenuated influenza virus strains make it appropriate to review and update the WHO recommendations for such vaccines. Since the requirements for influenza vaccine (live) were published in 1979, there have been significant advances in influenza virus vaccine production. For example, current reverse genetics techniques allow the selection of a homogeneous predefined viral composition by using a system that reconstitutes influenza viruses from genetic codes specific for each of the eight influenza viral gene segments (9–11). Knowledge of the genetic markers associated with virus attenuation has also increased, allowing more stringent control of the vaccine. Additionally, considerable efforts have been devoted to pandemic planning to ensure that safe and effective vaccines can be produced quickly in response to a pandemic emergency (12, 13). It is therefore necessary to revise the previous guidance on live attenuated influenza vaccines in order to reflect new developments and current practices in the field. In accordance with current WHO policy, the revised document is renamed as “Recommendations”.

### Part A. Manufacturing recommendations

#### A.1 Definitions

##### A.1.1 International name and proper name

The proper name of the vaccine shall be “influenza vaccine (human, live attenuated)”, translated into the language of the country of origin.

Use of the proper name should be limited to vaccines that satisfy the recommendations formulated below.
A.1.2  **Descriptive definition**  
Influenza vaccine (human, live attenuated) is a preparation of live attenuated influenza virus originating from human or other species. Influenza vaccine (human, live attenuated) is an aqueous suspension, which may be lyophilized, and is intended for intranasal administration. Influenza vaccines (human, live attenuated) contain a strain or strains of influenza virus types A or B or a mixture of these two types, which have been grown individually in embryonated hen’s eggs or in cell cultures. The influenza vaccines (human, live attenuated) shall be named “human” as they are to be administered to human beings.

A.1.3  **International standards**  
No international reference preparations are currently available for quality control or release testing of influenza vaccine (human, live attenuated) for intranasal administration. Moreover, these influenza vaccines may differ between manufacturers such that infectivity tests and/or potency assays may not be standardized for universal application. Therefore, no recommendations based on the use of international reference preparations can be formulated at present for influenza vaccine (human, live attenuated) for intranasal administration.

Each manufacturer of influenza vaccines (human, live attenuated) for intranasal administration should provide preparations of reference live attenuated influenza viruses and specific antisera for use in tests of virus infectivity and/or potency specific to the live attenuated vaccine (see sections A.5.3.1, A.5.4.1 and A.7.3). The manufacturer should cooperate with national regulatory authorities to determine the acceptability of the proposed reference reagents.

The infectivity tests and/or potency assays should be established and validated during vaccine development and approved by the national regulatory authority. Strains used for quality control or release testing of influenza vaccines (human, live attenuated) for intranasal administration should be preparations antigenically representative of viruses with surface antigens (haemagglutinin and neuraminidase) identical or closely related to the WHO-recommended vaccine strains (3).

A.1.4  **Terminology**  
The definitions given below apply to the terms used in these recommendations. They may have different meanings in other contexts.

**Adventitious agents.** Contaminating microorganisms of the cell culture or line including bacteria, fungi, mycoplasmas and viruses that have been unintentionally introduced (14). WHO is developing further guidance on adventitious agents.

**Attenuated donor virus strain.** An attenuated influenza virus that provides the genes of attenuation for vaccine strains that can be shown to be safe during clinical trials in human beings (15).
Candidate influenza vaccine viruses for seasonal or non-highly pathogenic influenza A subtype viruses with pandemic potential. These influenza viruses, approved by WHO as suitable for making influenza vaccine, are typically prepared in vaccine virus reassortment laboratories by “classical” reassortment; however, reverse genetics techniques may also be considered in their preparation (16).

Candidate influenza vaccine viruses for H5N1 and other highly pathogenic influenza A subtype viruses. These influenza viruses, approved by WHO as suitable for making influenza vaccine, are prepared in vaccine virus reassortment laboratories by reverse genetics (16).

Cell bank. A collection of ampoules containing material of uniform composition stored under defined conditions, each ampoule containing an aliquot of a single pool of cells (14).

Cell seed. A quantity of well-characterized cells of human, animal or other origin stored frozen at or below –100 °C 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 (14).

Classical genetics (classical reassortment). The process of finding and assembling a set of genes that affect a biological property of interest. In this process, mutants (reassortants) are generated by employing mutagens that accelerate the normal mutation rate or by growing the organism and allowing spontaneous mutations to occur. Mutants are selected for a particular biological property (phenotype) that differentiates them from the wild type. The location of the mutations responsible for the mutant phenotype is identified and analysed to determine the role of the altered DNA on the studied biological property (17).

Clinical evaluation of vaccines. Includes all the clinical trials and other clinical studies conducted in human beings pre- and post-licensure to determine the safe and effective use of vaccines intended for the control of specific diseases. Clinical evaluation is done in phases so that information is gathered in a coherent manner that respects the rights and dignity of all study participants and reduces the risks to participants, and that provides an understanding of the potential benefit of the vaccine under study (18).

Egg infectivity dose 50% (EID\textsubscript{50}). The quantity of a virus suspension that will infect 50% of embryonated hen’s eggs inoculated with the suspension (9).

Final bulk. The finished vaccine prepared from one or more monovalent pools present in the container from which the final containers are filled. It may contain one or more virus strains (2).

Final lot. A collection of sealed final containers that are homogeneous with respect to the risk of contamination during filling procedures (including lyophilization). A filling lot must therefore have undergone the filling procedures (including lyophilization) in one working session from a single final bulk (2).
**Genetic reassortment.** In genetic reassortment, genes from two or more influenza viruses are mixed in different combinations, resulting in hybrid viruses with genetic characteristics of each parent virus. This process occurs in nature but can also be achieved in a laboratory using “classical” reassortment or reverse genetics (16, 17).

**High-growth reassortant viruses.** Influenza viruses that have been genetically modified to grow better in embryonated hen’s eggs for optimal vaccine production (16).

**Highly pathogenic influenza viruses.** Influenza viruses (typically from an avian host) that cause at least 75% mortality when inoculated intravenously into 4–8-week-old chickens (19).

**Influenza reference viruses.** Wild-type influenza viruses selected by WHO as representative of important groups of influenza viruses on the basis of extensive antigenic and genetic studies and comparisons with viruses from many countries. As the influenza viruses evolve in nature, new reference viruses are selected (16).

**Influenza virus subtype(s).** Type A influenza viruses are further classified according to their combinations of haemagglutinin (H) and neuraminidase (N) antigens (i.e. specific proteins on the virus surface), e.g. H5N1. Sixteen H subtypes and nine N subtypes have been distinguished (16).

**Master cell bank.** A quantity of fully characterized cells of human or other animal origin derived from the cell seed stored frozen at or below −100 °C in aliquots of uniform composition derived from the cell seed. The master cell bank is itself an aliquot of a single pool of cells generally prepared from a selected cell clone under defined conditions, dispensed into multiple containers and stored under defined conditions. The master cell bank is used to derive all working cell banks. The testing performed on a replacement master cell bank (derived from the same cell clone or from an existing master or working cell bank) is the same as for the initial master cell bank, unless a justified exception is made (2).

**Master seed lot.** The virus used to prepare the master seed lot is an attenuated influenza virus that combines the attenuating features of attenuated virus donor strain and the immunizing features of the wild-type virus reference strain. The master seed lot is a virus preparation that is antigenically representative of a WHO-recommended strain that has been processed at one time to ensure a uniform composition, is fully characterized, and may be used for the preparation of working seed lots or for production of vaccine. The national regulatory authority approves the master seed lot and its passage level.

**Median tissue culture infective dose 50% (TCID\textsubscript{50}).** The quantity of a virus suspension that will infect 50% of tissue culture inoculated with the suspension.

**Monovalent virus pool.** A pool of a number of single harvests of a single virus strain processed at the same time (2).

**Nonclinical evaluation of vaccines.** All in vivo and in vitro testing performed before and during the clinical development of vaccines. The potential
toxicity of a vaccine should be assessed not only before the start of human trials but throughout clinical development (20).

Non-highly pathogenic influenza viruses. Influenza viruses (sometimes also termed low-pathogenic) that cause less than 75% mortality when inoculated intravenously into 4–8-week-old chickens (19).

Novel (new) subtype of human influenza A virus. Refers to human influenza viruses with haemagglutinin and/or neuraminidase antigens that are distinct from seasonal influenza viruses and with the potential to cause a pandemic (16).

Plaque-forming unit (pfu). The smallest quantity of a virus suspension that will produce a plaque in monolayer cell cultures (21).

Preclinical evaluation of vaccines. All in vivo and in vitro testing carried out before the first testing of vaccines in humans. This is a prerequisite for the initiation of clinical trials and includes product characterization, proof of concept/immunogenicity studies and animal safety testing (20).

Production cell cultures. A collection of cell cultures, used for biological production, that have been prepared together from one or more containers from the working cell bank or, in the case of primary cell cultures, from the tissues of one or more animals (14).

Reverse genetics. The technique of determining a gene's function by first sequencing and then mutating it, and identifying the nature of the change in the phenotype (17).

Single harvest. A quantity of virus suspension harvested from the growth substrate inoculated with the same virus strain and incubated and harvested together in one session (14).

Specific pathogen-free (SPF). Used in relation to animals that have been shown by the use of appropriate tests to be free from specified pathogenic microorganisms, and also refers to eggs derived from SPF birds (22).

Specific antibody-negative (SAN). Used in relation to animals that have been shown by the use of appropriate tests to be free from antibodies to specified avian pathogenic microorganisms, and also to eggs derived from SAN birds (22).

WHO-recommended viruses for vaccine use. Wild-type influenza viruses recommended by WHO as the basis for an influenza vaccine (16).

Wild-type influenza viruses. Influenza viruses that have been cultured either in eggs or in cells (i.e. isolated) directly from clinical specimens and that have not been modified (16).

Wild-type reference virus strain. An influenza virus that has been selected to antigenically represent the circulating viruses against which the vaccine should protect recipients (15).

Working seed lot. A quantity of fully characterized virus of uniform composition derived from a master seed lot by a number of passages not exceeding the maximum approved by the national regulatory authority. The working seed lot can be used for production of vaccines (2).
**Working cell bank.** A quantity of cells of uniform composition derived from the master cell bank at a finite passage level, dispensed in aliquots into individual containers appropriately stored, usually frozen at or below –100 °C, one or more of which would be used for production purposes. All containers are treated identically and, once removed from storage, are not returned to the stock (14).

**A.2 Background on influenza vaccine (human, live attenuated) production**

Even before inactivated influenza vaccines entered commercial use, there was interest in live attenuated vaccines. Motivation for their development included mimicking the immune responses to natural influenza virus infection.

A number of candidate attenuated virus donor strains have been examined over the years, including A/Puerto Rico/8/34 (H1N1), A/Okuda/57 (H2N2) (attenuated by simple serial passage), avian–human reassortant viruses (attenuated by host range characteristics of the avian donor strains), and temperature-sensitive mutants (attenuated by chance recovery of viruses, serial passage, or introduction of mutations by directed mutagenesis) (23, 24). Many candidates, however, have been withdrawn from consideration during clinical development. For example, reassortants prepared with A/Puerto Rico/8/34 are not always well attenuated and introduction of a temperature-sensitive mutation without other stabilizing mutations resulted in a virus strain prone to reversion to a non-attenuated form, which may occur after replication in a vaccine recipient.

Thus far, the most successful strategy for preparing attenuated virus donor strains has been the development of cold-adapted attenuated influenza viruses by serial passage at sequentially lower temperature, which produced mutations in multiple gene segments (25–29). The presence of multiple mutations involving several influenza virus gene segments appears to contribute to the stability of the live attenuated virus genomes and to lower the probability of a reversion to virulence. Methods other than cold adaptation are also being explored and are in the early stages of evaluation (30–32). If new live influenza vaccines are approved for human use, these present recommendations may be revised.

Influenza vaccines contain the antigens of one or more influenza A and B viruses that represent the wild-type influenza viruses prevalent in human populations. Influenza A viruses are separated into subtypes based on structurally and antigenically distinct haemagglutinins and neuraminidases. Influenza B viruses are not separated into subtypes, but they do have genetic lineages of haemagglutinins and neuraminidases, which may be antigenically distinguishable. Influenza A subtypes and influenza B lineages undergo progressive evolutionary changes of haemagglutinin and neuraminidase antigens (antigenic drift), which may reduce the efficacy of vaccines when the
Vaccines are inadequately matched to the prevalent viruses. For influenza A virus subtypes not previously circulating in human populations (antigenic shift), it is expected that vaccines will be effective only if they incorporate the antigens of the novel influenza A subtype.

The composition of influenza vaccines (human, live attenuated), like that of other influenza vaccines, is constantly under review to optimize the protective efficacy against prevalent epidemic strains. Accordingly, WHO publishes recommendations twice a year concerning the strains to be included, so that virus strains antigenically matched to circulating strains are included in vaccines manufactured for distribution in the northern and southern hemispheres (3).

Antigenic modifications in the haemagglutinin and neuraminidase molecules typically involve variation in surface amino acid residues in the region of the molecule furthest from the viral envelope. Future antigenic variations cannot be predicted because the mechanism of selection of antigenic variants (antigenic drift) is not known and several evolutionary pathways appear possible. Antigenic shifts (the appearance of new influenza A haemagglutinin subtypes) are also unpredictable.

In addition to antigenic drift and shift, there is another type of variation among influenza viruses caused by the preferential growth of virus subpopulations in different host cells in which the virus is cultivated. Influenza viruses grown in embryonated hen’s eggs often exhibit genetic, antigenic and biological differences from those isolated and maintained in mammalian cells. Sequence analyses of the haemagglutinin genes of egg-adapted variants show that human influenza viruses grown in eggs are less likely to maintain fidelity to the original sequence than the same viruses grown in mammalian cells. It is therefore important in vaccine preparation to ensure that antigenic changes in the haemagglutinin molecule do not impair the protective effects of the vaccine.

There is a long history of safety for egg-grown influenza virus vaccines (33, 34). However, it is known that influenza viruses grown in embryonated hen’s eggs can be contaminated with other viral agents. Although the use of eggs from flocks husbanded to meet agricultural health criteria (35) for freedom from specific pathogens may reduce the chances of introduction of a microbial agent, adventitious agents can be introduced from any egg source. The recommendations in this document have been revised in view of the findings with egg-grown viruses, the increasing use of mammalian cells for virus isolation and vaccine production, and the improved methods of detecting adventitious agents (14, 36). WHO is developing further guidance on testing adventitious agents.

There have been several influenza A virus pandemic alerts since 1997 (H5N1 subtype in 1997, 2003, 2005 and later years; H7N7 in 2003; and H9N2 in 1999) when avian influenza A viruses caused illness often serious enough to require hospitalization and to cause death in infected humans. In addition, the 2009 experience with pandemic influenza A (H1N1) demonstrates the
potential for non-avian influenza viruses to cause significant morbidity and mortality. These events illustrate the need for some flexibility and a variety of strategies for the production and clinical use of a vaccine in response to a pandemic. For example:

- it may be necessary to generate a vaccine virus from a highly pathogenic virus by reverse genetics;
- monovalent vaccines may be preferred; and
- two vaccine doses may be needed by all vaccine recipients.

Reflecting the special needs of an influenza pandemic, WHO has developed recommendations to assure the quality, safety and efficacy of the vaccine for pandemic situations (12).

The use of reverse genetics for vaccine virus development is relevant to both inter-pandemic and pandemic vaccines (15). Reverse genetics technology has already been introduced as a method for generating reassortants for manufacturing commercial influenza vaccines (human, live attenuated). It allows necessary genetic modifications such as removal of virulence motifs, and is therefore also used to produce reassortants for use in vaccines in the event of a pandemic. Reverse genetics technology involves transfecting mammalian cells with plasmids coding for influenza virus genes in order to produce a virus reassortant. Production of reassortants in this way is similar in concept to classical reassorting methods, but there are some important differences.

- The source of the influenza virus haemagglutinin and neuraminidase genes for reverse genetics is reduced in importance since the process of extraction of nucleic acid eliminates concerns about adventitious agents: an egg isolate, an isolate in cells not approved for human vaccine production, or a clinical specimen may all be adequate to provide the nucleic acid needed for the start of reverse genetics.

- The reverse genetic reassortant virus is generated in mammalian cells acceptable to national regulatory authorities.

- In some countries, a reassortant produced using reverse genetics is classified as a “genetically modified organism” and the vaccine should comply with national regulations or with WHO’s Biosafety risk assessment and guidelines for the production and quality control of human influenza pandemic vaccines (13).

Development of a new candidate attenuated donor virus should apply the knowledge gained in developing safe and immunogenic vaccines. Significant information has accumulated to indicate that not all attenuation methods result in donor vaccine virus strains of acceptable stability. For example, it is known that
an attenuated donor virus strain based on a mutation conferring temperature sensitivity alone can be expected to revert to a virulent form, even after a single passage in human recipients (26). Attenuation due to multiple mutations appears to be more stable and probably more useful for long-term implementation of influenza vaccines (human, live attenuated). Genetic stability and retention of key phenotypic features of the attenuated donor strain are extremely important quality characteristics. Assurance that reversion to virulence is unlikely to occur in humans should be established in preclinical and clinical studies and monitored carefully during the post-marketing period.

Multiple strategies for deriving candidate master seed and working seed viruses may be considered for seasonal vaccines and vaccines against influenza A subtype viruses not classified as highly pathogenic (e.g. pandemic influenza A (H1N1) 2009 virus). Only reverse genetics is appropriate for candidate vaccine viruses derived from highly pathogenic avian influenza viruses. However, both classical reassorting methods and reverse genetics techniques represent controlled methods to be considered for developing seed viruses once a satisfactory attenuated virus donor strain is obtained.

Classical reassortment between the attenuated virus donor strain and the new wild-type virus requires selection steps that may not always be readily successful in providing the construct of choice. However, classical reassortment can be an effective and relatively rapid method of producing attenuated seed viruses with wild parent surface antigens. General experience with live virus vaccines shows that candidate seed viruses derived by classical reassortment should be cloned at least three times by limiting dilution passage in SPF-SAN embryonated eggs or plaque purified in qualified cells to ensure purity of the desired attenuated seed virus. Reverse genetics methods permit a directed and more defined preparation of the desired reassortant virus. These methods also allow elimination of the highly pathogenic phenotype of avian influenza viruses, since the major molecular determinants of pathogenicity (multiple basic amino acids at the haemagglutinin cleavage site) can be removed during the preparation of the plasmids used to produce the reverse genetics reassortant. Neither reverse genetics nor classical reassortment eliminates the requirement for careful genetic and phenotypic assessment of the potential master seed and working seed viruses to ensure retention of attenuation.

Influenza viruses present a challenge for vaccine preparation as they exhibit continuous antigenic change in surface antigens of circulating influenza virus strains. Both inactivated and live influenza virus vaccines can confer a degree of cross-protection against related virus strains within a common haemagglutinin or neuraminidase subtype. For either type of vaccine, the use of a vaccine strain with haemagglutinin and neuraminidase antigenically identical to the naturally prevalent virus strain is expected to provide optimum protective
efficacy. In practice, however, it may not always be possible for the haemagglutinin and neuraminidase to be identical with influenza viruses, since further evolution may occur during the several months needed for vaccine preparation.

The interval between the appearance of an influenza virus variant and its spread throughout the world may be only a matter of months, and the development of an appropriate vaccine virus strain must therefore be rapid. The time available for the yearly preparation and testing of new influenza vaccine (human, live attenuated) lots must be carefully estimated and planned; some flexibility may be possible in completing control tests for routine production lots. Clinical studies, if undertaken, must be focused but the information they can provide about potential vaccine performance is likely to be severely limited.

Where appropriate, the technologies and experience acquired over several decades in the production and control of all live virus vaccines should be applied to influenza vaccines (human, live attenuated) for intranasal administration. As for all live vaccines, the substrate on which the virus is propagated is critically important to maintaining the consistency and safety of the vaccine product. Influenza vaccines (human, live attenuated) for intranasal administration are usually produced in 9–11-day-old vaccine-quality embryonated eggs; a large measure of safety can be assured by sourcing the eggs from closed-layer flocks that are continuously monitored for known specific pathogenic agents and antibodies against them. These SPF-SAN layer flocks are now available in several countries, and the eggs – or cell cultures derived from them – have been widely used in the production of a number of vaccines, including measles and mumps vaccines (37).

The production of influenza vaccines (human, live attenuated) using cell culture is currently under active research. When considering the use of animal cells as in vitro substrates for the production of biologicals, specific WHO guidelines should be taken into account (14, 36). Since live influenza vaccines depend on the viability of the virus in filled containers, the storage conditions as well as the short- and long-term stability of liquid and lyophilized products should be established by rigorous studies similar to those undertaken in the preparation of other live virus vaccines (38).

Apart from addressing the technical challenges associated with the rapid development of suitable attenuated vaccine seed viruses, manufacturers and national regulatory authorities should cooperate to define the need for special administrative arrangements for registration and licensing and to establish the nature of data needed for review of the vaccine product preparation and use. Close collaboration between manufacturers and the national regulatory authorities is required, particularly during development, production and testing of the initial batches of live vaccines. The time for considering the risks and benefits of the vaccines and for completing licensing procedures should be as short as practically possible if appropriate influenza vaccines (human, live attenuated) for intranasal administration are to be available soon after the emergence of a variant.
In a public health emergency, i.e. influenza pandemic, the abnormal vaccine demand may create difficulties if all tests recommended in the WHO guidelines on regulatory preparedness for human pandemic influenza vaccines (12) are to be carried out. Decisions to modify these requirements in the interests of public health during such an emergency are the responsibility of national regulatory authorities. Since progress in the development and implementation of live attenuated influenza vaccines may result in improvements or, conversely, in additional concerns to be addressed, the WHO Recommendations to assure the quality, safety, and efficacy of influenza vaccines (human, live attenuated) for intranasal administration are expected to need updating from time to time.

A.3 General manufacturing recommendations
The general requirements for manufacturing establishments contained in WHO good manufacturing practices for biological products (39) should apply to establishments manufacturing influenza vaccine (human, live attenuated) for intranasal administration, with the addition of the following.

A.3.1 Procedures and facilities
Details of standard operating procedures for the production and testing of influenza vaccines adopted by a manufacturer, together with evidence of appropriate validation of the production process, should be submitted to the national regulatory authority for approval. Proposals for modification of the manufacturing/control methods should also be submitted to the national regulatory authority for approval.

Production areas should be cleaned, disinfected and/or decontaminated by validated procedures before being used for the manufacture of influenza vaccines (human, live attenuated) for intranasal administration. The areas where processing of live attenuated influenza vaccines takes place, and the procedures used for manufacturing, should be designed to ensure that it is impossible to contaminate the influenza vaccine (human, live attenuated) for intranasal administration with another product. It is considered that filling of influenza vaccine (human, live attenuated) for intranasal administration could occur on a campaign basis in the same facility used for filling other vaccines provided that the manufacturer develops and performs a risk analysis and evaluation and puts in place validated procedures for risk control (13).

Facilities for vaccine production should be constructed with adequate containment features to accommodate the candidate influenza vaccines derived from the wild-type influenza viruses; WHO’s Biosafety risk assessment and guidelines for the production and quality control of human influenza pandemic vaccines (13) should be followed. Standard operating procedures must be developed for dealing with emergencies involving accidental spillage, leakage
or other dissemination of virus. High levels of biocontainment are required for work with the highly pathogenic wild-type influenza viruses that may be used in generating master and working seed viruses.

The production of influenza vaccines (human, live attenuated) for intranasal administration should be conducted by staff who have not handled other infectious microorganisms or animals on the same working day. Staff must practise good sanitation and health habits and personnel with respiratory or other apparent infectious illness should be excluded. Particular attention should be paid to the recommendations given in WHO good manufacturing practices for biological products (39) regarding the training and experience of personnel in charge of production and testing and of those assigned various responsibilities in the manufacturing establishment. Personnel employed in the production and control facilities should be adequately trained and protected against accidental infection with influenza virus according to guidance in the WHO biosafety manual (40) and in Biosafety guidelines for personnel engaged in the production of vaccines and biological products (41). Protection of personnel and containment measures should also follow Biosafety risk assessment and guidelines for the production and quality control of human influenza pandemic vaccines (13).

Manufacturers and national regulatory authorities should consider whether the influenza vaccine (human, live attenuated) for intranasal administration presents any significant environmental, agricultural, or human risks. WHO’s Biosafety risk assessment and guidelines for the production and quality control of human influenza pandemic vaccines (13) provides a detailed strategy to minimize the risks of introducing influenza virus strains into the community.

### A.3.2 Eggs and cell cultures

Fertile eggs are currently the preferred substrate for vaccine production, with an estimated 600 million eggs being used annually for this purpose worldwide. In general, two kinds of vaccine-quality embryonated eggs are available for the production of influenza vaccines (human, live attenuated) for intranasal administration – SPF-SAN and non-SPF eggs (42).

Only vaccine-quality embryonated hen’s eggs obtained from layer flocks meeting the health surveillance requirements of the relevant national animal health authority and the national regulatory authority for the production of influenza vaccine (human, live attenuated) for intranasal administration should be introduced into or handled in the production area.

General requirements for animal health surveillance have been established by the World Organisation for Animal Health (35). Internationally accepted requirements on hygiene and disease security procedures in poultry breeding flocks and hatcheries have also been established (43). The relevant national animal health authority and the national regulatory...
authority in Member Countries and Territories of the World Organisation for Animal Health (OIE) are bound to follow the OIE *Terrestrial animal health code* (35, 43). The relevant national animal health authority and the national regulatory authority should work together to establish national animal health requirements for layer flocks from which vaccine-quality embryonated eggs are obtained for production of influenza vaccines (human, live attenuated) for intranasal administration.

**Use of vaccine-quality SPF-SAN embryonated eggs**

The use of vaccine-quality SPF-SAN embryonated eggs is encouraged for the manufacture of influenza vaccines (human, live attenuated) for intranasal administration but this does not eliminate the need for adventitious agent (as defined in section A.1.4.) testing.

The animal health requirements for SPF-SAN layer flocks are similar across regions and countries (35, 43–45). Hens and roosters in SPF-SAN layer flocks are kept under strictly isolated conditions to guarantee freedom from the avian pathogens (SPF layer flocks) and antibodies (SAN layer flocks) against the avian pathogens that are laid down in the national animal health and regulatory requirements. These flocks are not vaccinated against avian pathogens and must be kept in filtered-air positive-pressure poultry housing in isolation from commercial poultry (37, 46).

A health surveillance programme in SPF-SAN layer flocks is strictly followed and tests are performed regularly to ensure the SPF and SAN status. In some countries, SPF-SAN layer flocks are monitored weekly for quality control: all birds are bled when an SPF-SAN layer flock is established, and thereafter a percentage of the birds are bled at specified intervals. The sera are screened for antibodies to the relevant avian pathogens. These pathogens may also be detected in the flocks by culture or other detection methods including polymerase chain reaction (PCR). Any death in an SPF-SAN layer flock is investigated to determine causality. Permanent records of mortality and results of layer flock testing are kept for several years (usually five). Egg users should be notified immediately when any test results indicate infection with a specified pathogen and when any deterioration in egg production or hatchability is observed in the source layer flock (47).

Layer flocks providing vaccine-quality SPF-SAN embryonated eggs for production of influenza vaccines (human, live attenuated) for intranasal administration should be evaluated frequently to detect exposure of the flock to avian pathogens, which have the potential to cause quality failure in assessments for adventitious agents (as defined in section A.1.4). The quality of SPF-SAN embryonated eggs varies according
to the extent of avian pathogen testing performed in the layer flocks (48). Avian pathogens of interest in SPF-SAN layer flocks may vary by geographical region (37, 43–45, 47) and include, as a minimum: avian adenoviruses, avian encephalomyelitis virus, avian infectious bronchitis viruses, avian infectious laryngotracheitis virus, avian leukosis viruses, avian nephritis virus, avian orthoreoviruses, avian reticuloendotheliosis virus, chicken anaemia virus, egg drop syndrome virus, fowlpox virus, infectious bursal disease viruses, influenza A viruses, Marek’s disease virus, Newcastle disease virus, *Mycobacterium avium*, *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, *Salmonella gallinarum*, *Salmonella pullorum*, *Salmonella* species, and *Haemophilus paragallinarum*.

If vaccine-quality SPF-SAN embryonated eggs are used for production of influenza vaccine (human, live attenuated) for intranasal administration, the manufacturer should ensure that layer flock health surveillance is consistent with the requirements of the relevant national animal health authority and the national regulatory authority. The SPF-SAN egg supplier should provide the manufacturer with a quality control certificate showing the testing methods used and the test results performed in accordance with the requirements of the relevant national animal health authority and the national regulatory authority.

The use of vaccine-quality embryonated eggs from SPF-SAN layer flocks for production of influenza vaccine (human, live attenuated) for intranasal administration is not a regulatory requirement in any country where such vaccine is currently manufactured.

**Use of vaccine-quality non-SPF embryonated eggs**

As a large number of embryonated eggs are needed for human influenza vaccine production, it may not always be feasible to use vaccine-quality embryonated eggs from SPF-SAN layer flocks. Nowadays, the largest volume of embryonated eggs used for human influenza vaccine production worldwide are vaccine-quality non-SPF (42, 49).

If vaccine-quality non-SPF embryonated eggs are used for production of influenza vaccines (human, live attenuated) for intranasal administration, the manufacturer should ensure that the layer flock health surveillance is consistent with the requirements of the relevant national animal health authority and the national regulatory authority (36, 42). The manufacturer should ensure that the non-SPF layer flocks are managed with strict attention to environmental cleanliness and control of access to the flock. The manufacturer should ensure that the vaccine-quality non-SPF embryonated eggs used for vaccine production are highly consistent in their physical and biological qualities, thereby meeting specified requirements of cleanliness and viability.
Hens and roosters in non-SPF flocks are kept under conditions similar to the parent flock for the production of day-old commercial layer chicks. The vaccination programme against common avian pathogens in non-SPF layer flocks has similarities by region and country and generally includes: Marek’s disease virus, *Salmonella* species, Newcastle disease viruses, avian infectious bronchitis viruses, Gumboro, avian infectious laryngotracheitis virus, avian encephalomyelitis virus, *Escherichia coli*, and chicken anaemia virus (if necessary).

Sera are collected throughout the life of the non-SPF layer flock and tested for antibodies against *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, *Salmonella* species, Newcastle disease viruses, avian infectious bronchitis viruses, avian reoviruses, avian adenoviruses, avian infectious laryngotracheitis virus, avian influenza viruses, avian encephalomyelitis virus, and chicken anaemia virus. Vaccine-quality non-SPF embryonated eggs can be produced free of antibodies for specific diseases by adjusting the vaccination programme of the source layer flock (35, 43).

If vaccine-quality non-SPF embryonated eggs are used for production of influenza vaccine (human, live attenuated) for intranasal administration, the manufacturer should ensure that the flock health surveillance programme is consistent with the requirements of the relevant national animal health authority and the national regulatory authority. The supplier of vaccine-quality non-SPF embryonated eggs should provide the manufacturer with a quality control certificate showing the vaccination programme and the tests carried out under their animal health surveillance programme. The control certificate should be in accordance with the requirements of the relevant national animal health authority and the national regulatory authority.

There should be much greater scrutiny of adventitious agent (as defined in section A.1.4) testing when vaccine-quality non-SPF embryonated eggs are used throughout the vaccine development and production process. If the vaccine strain is produced in vaccine-quality non-SPF embryonated eggs, the national regulatory authority and national control laboratory should specify the additional tests for the detection of adventitious agents that could be derived from the substrates used in preparation of the donor virus, seed virus strains, and vaccine virus. Knowledge of local, regional, and national zoonotic avian diseases would aid decision-making on adventitious agents testing for influenza vaccines (human, live attenuated) for intranasal administration (50, 51). Moreover, knowledge of the vaccination and antibody testing programmes of non-SPF layer flocks required by the relevant national animal health authority and the national regulatory authority should provide a foundation for sound decisions on what adventitious agents to test when vaccine-quality non-SPF embryonated eggs are used.
Only cell cultures meeting the requirements of the national regulatory authority should be used in the production of influenza vaccine (human, live attenuated) for intranasal administration. Cell cultures should also conform to the principles established in Requirements for the use of animal cells as in vitro substrates for the production of biologicals (14, 36).

A.4 **Control of source materials**

A.4.1 **Choice of vaccine strain**

The World Health Organization, with the participation of WHO collaborating centres and WHO essential regulatory laboratories, reviews the global influenza epidemiological situation twice annually and recommends influenza reference viruses for the composition of seasonal influenza vaccines for the northern and southern hemispheres in accordance with available evidence (3). With the participation of WHO collaborating centres, WHO essential regulatory laboratories and WHO H5 reference laboratories, the Organization also reviews and reports the circulation of wild-type influenza viruses of pandemic potential, as well as the development status of candidate influenza vaccine viruses to be used for pandemic preparedness (3).

Preparation of the live attenuated vaccine master seed virus is typically the responsibility of the laboratories engaged in production of a specific influenza vaccine (human, live attenuated) for intranasal administration. The preparing laboratory uses an attenuated master donor virus (which contributes the essential attenuating genes) and a wild-type influenza virus reference strain to derive a reassortant virus. The reassortant virus is fully characterized to determine whether it has the proper genetic and phenotypic properties; if it is satisfactory, it becomes a master seed virus, which may be used to produce a working seed virus and vaccine.

Although master and working seed viruses for an influenza vaccine (human, live attenuated) for intranasal administration are typically used exclusively by the preparer, it is possible in some circumstances for a seed virus to be provided to other manufacturers. In this case, it is expected that validated quality control procedures would be in place at the receiving manufacturer in order adequately to identify the genetic characteristics, as well as the antigenic and attenuating properties, of the seed virus before its use in vaccine production.

Influenza vaccines (human, live attenuated) for intranasal administration should include the surface glycoproteins (haemagglutinin and neuraminidase) of the influenza virus reference strains recommended by WHO for inclusion in vaccines (3), or from strains antigenically closely related to them, as approved by the national regulatory authority.
Influenza reference viruses for antigenic analysis and for preparation of reassortant viruses may be obtained from the WHO Collaborating Centres for Reference and Research on Influenza or other custodian laboratory (see Appendix 2).

In some years, the specific influenza reference viruses used for the preparation of influenza vaccines (human, live attenuated) and influenza vaccines (inactivated) differ, but the vaccine viruses in any case are antigenically representative of the WHO-recommended influenza reference viruses (3).

The passage history of the parental and reassortant viruses, together with full documentation of the characterization of the genetic and phenotypic properties of the master seed virus, should be submitted to the national regulatory authority for approval.

A.4.2 Substrate for virus propagation

A.4.2.1 Eggs used for seed virus growth

Vaccine seed virus is produced in vaccine-quality embryonated eggs from healthy layer flocks that are monitored by methods approved by the relevant national animal health authority and the national regulatory authority (see section A.3.2).

In some countries, vaccine-quality 9–11-day-old embryonated eggs are used.

In production of vaccine seed virus, the egg source layer flock should not have been vaccinated with live Newcastle disease virus vaccine. In addition, layer flocks should not be receiving any chemotherapeutic agents (e.g. antimicrobial agents or coccidiostats). It is also recommended that vaccine-quality embryonated eggs be obtained from young hens.

In countries where use of live Newcastle disease vaccine or any other live vaccine is mandatory, vaccination should take place during the first few weeks of the hen’s life and well before the use of flocks for egg supply.

Hence, the use of vaccine-quality SPF-SAN embryonated eggs is required for growth of the seed virus for influenza vaccine (human, live attenuated) for intranasal administration (see section A.3.2).

A.4.2.2 Eggs used for vaccine production

Vaccine is produced in vaccine-quality embryonated eggs from healthy layer flocks that are monitored by methods approved by the relevant animal health authority and the national regulatory authority (see section A.3.2).
In some countries, vaccine-quality 9–11-day-old embryonated eggs are used.

In production of vaccine, the egg source layer flock must not have been vaccinated with live Newcastle disease virus vaccine. In addition, layer flocks should not be receiving any chemotherapeutic agents (e.g. antimicrobial agents or coccidiostats). It is also recommended that vaccine-quality embryonated eggs be obtained from young hens.

In countries where use of live Newcastle disease vaccine or any other live vaccine is mandatory, vaccination should take place during the first few weeks of the hen’s life and well before the use of flocks for egg supply.

Hence, the use of vaccine-quality SPF-SAN embryonated eggs is encouraged for the manufacture of influenza vaccine (human, live attenuated) for intranasal administration (see section A.3.2).

A.4.3 Master cell bank and manufacturer’s working cell bank

A.4.3.1 Cell bank system

A cell line used for the manufacture of influenza vaccine (human, live attenuated) for intranasal administration should be based on a cell bank system. The national regulatory authority should approve the master cell bank and should establish the maximum number of passages (or population doublings) by which the manufacturer’s working cell bank is derived from the master cell bank and the maximum number of passages (or population doublings) of the production cultures.

WHO has established a reference cell bank of Vero cells characterized in accordance with Requirements for the use of animal cells as in vitro substrates for the production of biologicals (14, 36). Those requirements may be revised during the lifespan of this document on influenza vaccines (human, live attenuated) for intranasal administration: manufacturers and national regulatory authorities are encouraged to monitor WHO publications for corresponding updates (http://www.who.int/immunization/en/).

The WHO 10-87 cell bank of Vero cells is stored at the European Collection of Animal Cell Cultures (ECACC), Porton Down, England, and at the American Type Culture Collection (ATCC), Rockville, MD, USA. This cell bank should not be considered as the master cell bank for direct use in vaccine production but may be used to establish master cell banks for thorough requalification. Producers of biologicals and national regulatory authorities can obtain culture of these Vero cells (free of charge), as well as additional background information, from Quality, Safety and Standards of Biologicals, World Health Organization, 1211 Geneva 27, Switzerland.
A.4.3.2 Identity test

The master cell bank should be characterized according to WHO’s Requirements for the use of animal cells as in vitro substrates for the production of biologicals (14, 36) as they relate to continuous cell lines, or to human diploid cells, as appropriate.

The manufacturer’s working cell bank should be identified by means, among others, of biochemical (e.g. isoenzyme analysis), immunological and cytogenetic marker tests, and DNA fingerprinting approved by the national regulatory authority and the national control laboratory.

A.4.4 Cell culture medium

At every stage of preparation of donor viruses and vaccines, the sera used for propagation of cells (including the donor strains, master seed lot, working seed lot, master cell bank, working cell bank and production cell cultures) should be tested for freedom from bacteria, fungi and mycoplasma according to the WHO General requirements for the sterility of biological substances (52).

Sera used in the propagation of cells should also be tested for freedom from adventitious agents according to WHO Requirements (14, 36). Manufacturers are encouraged to explore the possibilities of using serum-free media for the production of influenza vaccines (human, live attenuated) for intranasal administration.

Suitable tests for detecting bovine viruses in serum, using either primary bovine testis cells or continuous bovine kidney-cell lines known to be sensitive to bovine viruses, are given in Appendix 1 of Recommendations for the production and control of poliomyelitis vaccine (oral) (53, 54).

Where approved by the national regulatory authority and the national control laboratory, alternative tests for bovine viruses may be used. As an additional monitor of quality, sera may be examined for freedom from phage and endotoxin.

The source(s) of serum of bovine origin should be approved by the national regulatory authority. The serum should comply with the WHO guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products (55).

Human serum should not be used. If human serum albumin is used, it should meet the Requirements for the collection, processing and quality control of blood, blood components and plasma derivatives (56) and comply with the WHO guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products (55).

Penicillin and other beta-lactam antimicrobial agents should not be used at any stage of manufacture.
Other antimicrobial agents may be used at any stage of manufacture, provided that the quantity present in the final product is acceptable to the national regulatory authority. 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 national regulatory authority may be added.

Trypsin used for preparing the master cell bank, working cell bank and production cell cultures should be tested and demonstrated to be free from cultivable bacteria, fungi, mycoplasmas and infectious viruses, especially paroviruses appropriate to the species of animal used. The methods used to ensure this should be approved by the national regulatory authority and the national control laboratory.

The source of trypsin of bovine origin, if used, should be approved by the national regulatory authority. Bovine trypsin, if used, should comply with current WHO guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products (55).

A.4.5 Virus strains
A.4.5.1 Origin and preparation of virus strains

Strains of influenza virus used in the production of influenza vaccines (human, live attenuated) for intranasal administration should be identified by historical records, which should include information on the origin of the donor virus strains, both the attenuated virus donor strain and the wild-type virus reference strain, as well as on the process used for preparing the attenuated master seed lot and working seed lot.

Only strains that have been isolated in vaccine-quality embryonated eggs (see section A.3.2), in cells derived from eggs or in mammalian cells approved by the national regulatory authority for human vaccine production should be used (unless reverse genetics technology is being used for preparation of the seed lot, as described later in this section). The national regulatory authority should also approve the attenuated virus donor strain and wild-type virus reference strain used for preparing the master seed lot. The vaccine seed viruses should have the attenuation phenotype of the attenuated donor virus strain, and the surface antigens (haemagglutinin and neuraminidase) should correspond to the influenza reference viruses recommended by WHO for vaccine preparation (3).

Experience shows that candidate seed viruses derived by classical reassortment should be cloned at least three times by limiting dilution passage in vaccine-quality SPF-SAN embryonated eggs or plaque purified in qualified cells to ensure purity of the desired attenuated seed virus. Since antigenic changes are possible during the development of reassortant viruses, the absence of antigenic changes in the master seed lot and working seed lot should be demonstrated at least by haemagglutination inhibition tests using antibodies to the haemagglutinins of the candidate influenza vaccine virus and of the wild-type reference virus strain.
Where reassortant viruses are used, the method for producing the reassortant should be approved by the national regulatory authority. Preparation of the live attenuated vaccine master seed viruses are typically the responsibility of the laboratories engaged in production of specific influenza vaccines (human, live attenuated) for intranasal administration.

Master and working seed viruses for an influenza vaccine (human, live attenuated) for intranasal administration are typically used exclusively by the preparer. If a master or working seed virus is provided to another manufacturer, the receiving manufacturer should use validated methods to identify the genetic characteristics and the antigenic and attenuating properties of the seed virus before using it in vaccine production. The receiving manufacturer should also have current good manufacturing practices (cGMP) in place to handle the master seed.

Where reverse genetics techniques are used to generate the reassortant vaccine virus, the influenza haemagglutinin and neuraminidase genes may be derived from a variety of sources (egg isolate, mammalian cell isolate or virus in clinical specimen). With reverse genetics, the source of viral genes is less critical than for classical reassortment because the haemagglutinin and neuraminidase genes are expected to be free of adventitious agents associated with wild-type virus by virtue of the recombinant DNA technology employed (15).

The cell substrate used for transfection to generate the reassortant virus by reverse genetic techniques should be appropriate for human vaccine production and approved by the national regulatory authority. The overall process for derivation of the reassortant virus prepared by reverse genetics should be approved by the national regulatory authority. WHO guidance on development of influenza vaccine reference viruses by reverse genetics (15) and WHO Guidelines for assuring the quality of pharmaceutical and biological products prepared by recombinant DNA technology (57) should be considered.

For work with highly pathogenic wild-type influenza viruses and newly emerging pandemic viruses, higher levels of biocontainment are required; Biosafety risk assessment guidelines for the production and quality control of human influenza pandemic vaccines (13) should be followed. Facilities for vaccine production should provide containment features to accommodate the candidate influenza vaccines that are derived from the wild-type influenza viruses.

If any materials of animal (non-avian) origin are used in vaccine production, they should comply with the WHO guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products (55) and should be approved by the national regulatory authority.

Reference strains for antigenic analysis may be obtained from WHO Collaborating Centres for Reference and Research on Influenza (see Appendix 2) or from another custodian laboratory.
A.4.5.2 **Seed lot system**

The production of vaccine should be based on a virus seed lot system. The influenza virus contained in each seed lot should be identified as the appropriate strain by methods acceptable to the national regulatory authority. The maximum number of passages between a master seed lot and a working seed lot should be approved by the national regulatory authority. The virus in the final vaccine should be not more than one passage removed from the working seed lot. The haemagglutinin and neuraminidase of the seed lot viruses should be identified by suitable tests.

The master seed lot may be considered for use as a working seed lot with the approval of the national regulatory authority. If the master seed lot is used as the working seed lot, the final vaccine should be not more than one passage removed from the master seed lot.

A.4.5.3 **Tests on seed lots**

A.4.5.3.1 **Adventitious agents**

The master seed lot and working seed lot should be shown to be free from relevant adventitious agents (as defined in section A.1.4) by tests or procedures approved by the national regulatory authority and the national control laboratory in accordance with the WHO General requirements for the sterility of biological substances (52, Part A, sections 5.2 and 5.3; 58).

Strategies to ensure freedom from adventitious agents (as defined in section A.1.4) in the final vaccine involve a combination of testing the seed virus and validation of the production process which depends on the substrate used for production and on the process developed for vaccine manufacture.

**Validation of processes**

Since the manufacturing of influenza vaccine (human, live attenuated) for intranasal administration is unlikely to include processes that are effective in inactivating potential contaminating agents, reliance is placed primarily on general precautions against microbial contamination in manufacture, on the quality of materials used for manufacture, and on testing for adventitious agents (52, 58). In some instances, removal of contaminating agents may be possible. For example, filtration steps may be used to remove bacteria or fungi derived from eggs. The production process should be validated, including steps designed for removal of potential contaminating microbial agents. Process validation may be performed with appropriate model agents. If removal of a potential contaminant cannot be demonstrated and validated, a testing strategy should be implemented to document the ability of individual steps and the overall process to prevent the introduction of potential microbial contaminating agents (52, 58).
Cells used to prepare vaccine

The susceptibility of cell cultures to various human pathogens should be taken into account and used in considering a list of potential human pathogens to be included in testing for adventitious agents in master seed lots and working seed lots passaged in the cells. Pathogens to be considered include adenovirus, parainfluenza virus, respiratory syncytial virus, coronavirus, rhinovirus, enterovirus, human herpesvirus 4 (Epstein–Barr virus), herpes simplex virus, cytomegalovirus and mycoplasmas (14, 36).

It is recognized that, when a vaccine strain changes, there may be time constraints that make testing master seed lots and working seed lots for adventitious agents problematic, and the full results of such testing may not always be available before further processing. The development and use of properly validated rapid assays such as immunoassays or PCR is therefore encouraged.

If an adventitious agent is detected in a master seed lot and/or working seed lot prepared in cell culture, those lots should not be used for vaccine production.

Embryonated eggs used to prepare vaccine

Whether vaccine-quality SPF-SAN or non-SPF embryonated eggs are used for vaccine preparation, a judicious testing strategy for specific potential adventitious agents is needed (see section A.3.2). The use of properly validated rapid assays such as immunoassays or PCR is encouraged.

The use of vaccine-quality SPF-SAN embryonated eggs does not eliminate the need for adventitious agent testing. However, there should be much greater scrutiny of adventitious agent testing when vaccine-quality non-SPF embryonated eggs are used for passage of the attenuated virus donor strain, wild-type virus reference strain, master seed lot, working seed lot or vaccine. In this case, the national regulatory authority should specify any additional tests for the detection of adventitious agents that could be derived from the substrates used in preparation of the master seed and working seed lots and in preparation of the vaccine (see section A.3.2).

If a potential contaminating agent is detected, the virus master seed lot or virus working seed lot should not be used for vaccine production.

A.4.5.3.2 Attenuation

Retention of key genetic and phenotypic characteristics related to attenuation may be demonstrated for either the master seed lot or the working seed lot of a virus strain to be introduced into clinical use.
The methods used to demonstrate attenuation may vary for each vaccine virus but should include at least one of the following:

- the full genetic sequence of the virus master seed lot or virus working seed lot,
- in vitro tests for the key phenotypic markers of the attenuated virus donor strain, and
- studies in an appropriate animal model to assess the in vivo aspects of vaccine virus attenuation.

Specific tests used to assess virus attenuation should be approved by the national regulatory authority and the national control laboratory.

The development of influenza vaccine (human, live attenuated) for intranasal administration should include an early assessment of the biosafety level required to work with the attenuated donor virus and the master seed viruses derived from the donor. Direct handling of an established attenuated donor virus strain for which much experience exists (such as the cold-adapted H2N2 strains) and of master seed viruses prepared for seasonal influenza vaccine viruses can generally be done using established biosafety level 2 (BSL-2) practices (13, 40, 59).

Work with highly pathogenic wild-type influenza viruses and newly emerging pandemic viruses requires higher levels of biocontainment, and the WHO Biosafety risk assessment and guidelines for the production and quality control of human influenza pandemic vaccines (13) should be followed. Facilities for vaccine production should provide adequate containment features to accommodate the candidate influenza vaccines that are derived from wild-type influenza viruses. Guidance for safe handling of influenza viruses is subject to revision based on evolving experience, and the biosafety level to be used should be decided in consultation with the national regulatory authority.

For candidate influenza vaccine viruses prepared using highly pathogenic avian influenza viruses as the starting material, additional tests should be performed. Optimally, the strategy to demonstrate elimination of highly pathogenic characteristics includes assessment of the ability of the candidate influenza vaccine virus to produce plaques in cell culture with and without trypsin, the ability to cause chick embryo death, pathogenicity in chickens (as defined in section A.1.4), and attenuation in ferrets (13). Specific tests used to assess the removal of highly pathogenic features should be approved by the national regulatory authority and the national control laboratory.

For candidate influenza vaccine viruses prepared using non-highly pathogenic influenza viruses of animal origin (avian, swine, equine, canine, others), additional safety tests may also be needed as described in WHO published guidance (13). Specific tests used to assess the candidate influenza
vaccine viruses should be approved by the national regulatory authority and the national control laboratory.

The virus seed lot should be stored at a temperature below –60 °C unless it is in the lyophilized form, in which case it should be stored at a temperature lower than –20 °C.

A.5 Control of vaccine production
A.5.1 Production precautions
The general production precautions formulated in the WHO good manufacturing practices and quality assurance for biological products should be followed (39, 57, 60). Although the WHO Recommendations for the production and control of influenza vaccine (inactivated) (2) are similar in many ways, the manufacture of influenza vaccines (human, live attenuated) for intranasal administration has important differences. The following should be observed.

- For embryonated egg-derived vaccines, only allantoic and amniotic fluids may be harvested.
- Beta-lactam antimicrobial agents should not be used at any stage in the manufacture of the vaccine and should not be permitted to come into contact with any part of the production equipment.

Minimal concentrations of other suitable antimicrobial agents may be used.

Small quantities of antimicrobial agents other than beta-lactam agents may be added with the approval of the national regulatory authority. However, if an antimicrobial agent is to be added, samples for sterility testing should be taken before the antimicrobial agent is added.

A.5.2 Production of monovalent virus pool
A.5.2.1 Single harvests
For egg-derived vaccines, each strain of virus should be grown in the allantoic cavity of vaccine-quality embryonated eggs derived from healthy layer flocks (see section A.3.2). After incubation at a controlled temperature, both the allantoic and amniotic fluids may be harvested to prepare a single harvest.

In some countries, vaccine-quality 9–11-day-old embryonated eggs are used.

It is recognized that adventitious agents (as defined in section A.1.4) can be introduced at any point in the process. It is a wise precaution to pool the allantoic fluids from a limited number of eggs (e.g. 30–50) and to test these small pools for sterility and virus titre before blending.
into the single harvest. The pools should be stored at a temperature of 2–8 °C. In case any contamination is detected in downstream processes, the manufacturer can test this small allantoic fluid pool for adventitious agents and virus titre to identify where the contamination or deterioration of virus titre occurred.

The use of vaccine-quality SPF-SAN embryonated eggs does not eliminate the need for adventitious agent testing. If the vaccine is prepared in vaccine-quality SPF-SAN embryonated eggs, it may be possible to obtain single harvests that are free of adventitious agents. However, in the event that this is not achievable, a bioburden limit on single harvests may be considered with the approval of the national regulatory authority.

There should be much greater scrutiny of adventitious agent testing when vaccine-quality non-SPF embryonated eggs are used to produce single harvests. If vaccine-quality non-SPF embryonated eggs are used, the national regulatory authority should specify any additional tests for the detection of the adventitious agents that could be derived from the egg substrates (see section A.3.2) used in preparation of attenuated virus donor strains, wild-type virus reference strains, virus master seed lot and virus working seed lots and in preparation of the vaccine.

For influenza vaccines (human, live attenuated) for intranasal administration prepared in cell cultures, each virus strain should be grown in cells approved by the national regulatory authority.

For both egg-derived and cell-derived vaccines, a number of single harvests of the same virus strain may be combined to give a monovalent virus pool. Cell-derived monovalent virus pools should not be mixed with egg-derived monovalent virus pools.

A.5.2.2 Tests of control eggs or cell cultures

The national regulatory authority should determine the need for control samples, the sample size to be examined, the time at which the control samples should be taken during the production process, and how the control samples are to be maintained.

When vaccine-quality embryonated eggs are used, a portion (2% or at least 20 eggs, whichever is the greater quantity) of each batch of the eggs used for vaccine virus propagation is held as uninoculated controls. These control eggs are incubated for the same time and at the same temperature and humidity as the inoculated embryonated eggs. At the time of harvesting the virus from the inoculated embryonated eggs, allantoic fluids are also taken from the uninoculated control eggs and examined for the existence of haemagglutinating agents (see section A.5.2.2.1).
To ensure freedom from adventitious agents (as defined in section A.1.4), the greatest reliance is placed on continuous health monitoring of the layer flock from which the vaccine-quality embryonated eggs are obtained (see section A.3.2).

The use of vaccine-quality SPF-SAN embryonated eggs does not eliminate the need for adventitious agent testing. There should be much greater scrutiny of adventitious agent testing when vaccine-quality non-SPF embryonated eggs are used.

The national regulatory authority and the national control laboratory should approve alternative strategies and methods for ensuring freedom from adventitious agents.

The national regulatory authority and the national control laboratory may include additional tests for microorganisms if necessary. Tests for avian leukosis virus in embryonated eggs are considered essential.

When testing is performed on control cell cultures, a sample equivalent to at least 500 ml of the cell culture suspension, kept at the same cell concentration as that used for vaccine production, is tested. Control cell cultures are incubated for at least two weeks and are examined during this observation period for evidence of cytopathic changes. For the test to be valid, not more than 20% of the control cultures may have been discarded for nonspecific reasons.

If any test described in this section yields evidence of the presence of an adventitious agent in a control cell culture or a control embryonated egg, the influenza virus grown in the corresponding inoculated cultures or eggs should not be used for vaccine production.

Samples not tested immediately should be stored at or below –60 °C.

**A.5.2.2.1 Tests for haemagglutinating and haemadsorbing agents**

For influenza vaccine (human, live attenuated) for intranasal administration prepared in vaccine-quality embryonated eggs, a sample of 0.25 ml of allantoic fluid taken from each control egg should be tested for haemagglutinating agents by the addition of chick erythrocytes, both directly and after one passage of the control allantoic fluid through vaccine-quality SPF-SAN eggs. The details of the test should be approved by the national regulatory authority and the national control laboratory.

For influenza vaccine (human, live attenuated) for intranasal administration prepared in cell culture, testing for the presence of haemadsorbing viruses at the end of the observation period or at the time the virus is harvested from the production substrate, whichever is later, should include at least 25% of control cells. The control cells should be tested using guinea-pig red blood
cells; if the red blood cells have been stored, the duration of storage should not have exceeded 7 days, and the storage temperature should have been in the range 2–8 °C. In testing for haemadsorbing viruses, calcium and magnesium ions should be absent from the medium.

This test is usually done using a 1% suspension of guinea-pig red blood cells. In some countries, however, the national regulatory authority requires that additional tests for haemadsorbing viruses be made in other types of red blood cells, including those from humans (blood group O), monkeys and chickens (or other avian species).

The results of all tests should be read after incubation for 30 minutes at 0–4 °C and again after a further incubation for 30 minutes at 20–25 °C. For tests using monkey red blood cells, a further reading should also be taken after incubation for 30 minutes at 34–37 °C.

In some countries the sensitivity of each new batch of red blood cells is demonstrated by titration against a haemagglutinin antigen before use in the haemadsorption test.

A.5.2.2.2 Tests on supernatant fluids from cell cultures

Samples of at least 10 ml of the pooled supernatant fluid from the control cell cultures collected at the end of the observation period should be tested for the presence of adventitious agents (as defined in section A.1.4) in monolayers of three indicator cell lines:

- cultures of cells of the same species and tissue type as those used for production;
- cultures of a human diploid cell line;
- cultures of another cell line from a non-human species.

The samples should be inoculated into containers of these indicator cell cultures, in such a way that the dilution of the supernatant fluid in the nutrient medium does not exceed 1 in 4. The area of the indicator cell sheet should be at least 3 cm²/ml of supernatant fluid. At least one container of each of the cell cultures should remain uninoculated and serve as a control.

The cultures should be incubated at a temperature of 35–37 °C and observed for cytopathic effect (and other evidence of a replicating adventitious agent) in the indicator cells for a period of at least two weeks.

The use of properly validated rapid assays such as immunoassays or PCR that could be conducted within the time constraints of the procedure is encouraged.
A.5.2.2.3 Identity test for continuous cell culture

For monovalent virus pools produced in continuous cell culture, the control cells should be identified by means approved by the national regulatory authority and the national control laboratory. Biochemical (e.g. isoenzyme analysis), immunological and/or cytogenetic marker tests may be considered for confirming identity.

A.5.2.3 Clarification and purification

The monovalent virus pool should be clarified and purified by centrifugation and/or other suitable methods approved by the national regulatory authority.

The aim of clarification and purification is to remove cell debris. For vaccine prepared in vaccine-quality embryonated eggs, a sterile filtration step may be considered to reduce bioburden. It is advisable to clarify and purify the influenza virus under conditions optimized for preserving its infectivity and antigenic properties.

Sterile filtration should be validated.

A.5.3 Control of monovalent virus pools

At the time when allantoic or tissue culture fluids are pooled to prepare the monovalent virus pool and before clarification and purification, samples should be set aside for examination for adventitious agents (as defined in section A.1.4). If the samples are not tested immediately, they should be stored at or below –60 °C.

For the purposes of the tests recommended in this section to verify neutralization of virus harvest, hyperimmune antibody preparations should be of an origin that will not cross-react with the antigens of cells or eggs used in production of the monovalent virus pool. The virus used for the production of the hyperimmune antibody preparations should be grown either in non-avian cell cultures or in vaccine-quality SPF-SAN embryonated eggs. If vaccine-quality embryonated eggs are used, they should be obtained from a different layer flock from that used to supply the vaccine production embryonated eggs.

For vaccines intended for use in a pandemic situation, hyperimmune sera for tests described in this section may not be readily available when they are needed for release and distribution of vaccines. As an alternative for both pandemic and non-pandemic situations, a strategy to identify potential contaminating agents by PCR methods may be considered. Alternative strategies should be approved by the national regulatory authority and the national control laboratory.
A.5.3.1 **Infectivity (potency)**

The influenza virus content in the clarified monovalent virus pool should be determined by titration in vaccine-quality embryonated eggs or cell cultures. The number of infectious doses of vaccine should be expressed in EID₅₀, TCID₅₀ or pfu per unit volume (see section A.7.3).

Additional equivalent methods dependent on the replication of virus in embryonated eggs or cell cultures (e.g. fluorescent focus assay expressed as fluorescent focus units) may be considered for determining potency.

A.5.3.2 **Attenuation**

Retention of key genetic and phenotypic characteristics related to attenuation of the candidate influenza vaccine virus should be demonstrated for the monovalent virus pool of a strain to be introduced into clinical use. The methods used to demonstrate attenuation of the monovalent virus pool may vary (60) and may include at least one of the following:

- the full genetic sequence of the virus of the monovalent virus pool,
- in vitro tests of the monovalent virus pool for the key phenotypic markers of the attenuated virus donor strain, and
- studies in an appropriate animal model (e.g. ferrets) to assess the in vivo aspects of attenuation of the vaccine preparation (13).

Attenuating features of the vaccine virus in the monovalent pool are expected to be identical with those of the seed virus (see section A.4.5.3.2).

A.5.3.3 **Identity**

Each monovalent virus pool lot should be identified as containing live attenuated influenza virus of the appropriate strain by methods acceptable to the national regulatory authority.

A.5.3.4 **Adventitious agent tests**

The tests described in this section form the traditional basis for identifying adventitious agents (as defined in section A.1.4). For vaccines intended for use in a pandemic situation, hyperimmune sera needed for tests in vaccine-quality embryonated eggs or cell cultures described in this section may not be readily available when they are needed for release and distribution of vaccines. More recent developments involving PCR methods therefore need to be considered, both to improve the specificity and sensitivity of adventitious agent testing and to anticipate situations in which delays in vaccine availability would be detrimental. The national regulatory authority and the national control laboratory should approve the specific methods used to fulfil adventitious agent testing.
The use of vaccine quality SPF-SAN embryonated eggs does not eliminate the need for adventitious agent testing. There should be much greater scrutiny of adventitious agent testing when vaccine-quality non-SPF embryonated eggs are used.

The monovalent virus pool passes if there is no evidence of any adventitious agent attributable to the virus pool.

A.5.3.4.1 Tests in vaccine-quality embryonated eggs

For vaccines produced in vaccine-quality embryonated eggs, a sample of at least 10 ml of each monovalent virus pool should be tested for the presence of adventitious agents (as defined in section A.1.4) by inoculation of eggs. After neutralization of the influenza virus by hyperimmune antibody preparation, the monovalent virus pool should be inoculated in vaccine-quality embryonated eggs. At least 0.25 ml of the virus/antibody mixture per egg should be used for inoculation of one group of eggs by the allantoic route and a separate group of eggs by the yolk sac route. The national regulatory authority should approve the method of incubation of the embryonated eggs and the observation time. None of the embryonated eggs should show evidence of the presence of any adventitious agents.

A.5.3.4.2 Tests in cell cultures

For vaccines produced in embryonated eggs or cell cultures, a sample of at least 5 ml of the monovalent virus pool should be tested for freedom from adventitious agents (as defined in section A.1.4) using cell cultures as described below. After neutralization of the influenza virus by hyperimmune antibody preparation, the monovalent virus pool should be inoculated on cell cultures of human cells, simian cells, chicken cells, or cells of the species used for vaccine production. The national regulatory authority should approve the cell cultures, the method of incubation and the period of observation. None of the cell cultures should show evidence of the presence of any adventitious agents. When chicken cells are used for vaccine production, the absence of avian leukosis viruses should be ascertained by testing.

A.5.3.4.3 Tests for bacteria, fungi and mycoplasma

The monovalent virus pool should be tested for freedom from bacteria, fungi and mycoplasma according to the WHO General requirements for the sterility of biological substances (52, 58).

A.5.3.4.4 Test for mycobacteria

Each monovalent virus pool should be tested for the presence of mycobacteria by methods appropriate for the detection of the organisms most likely to be found in the embryonated eggs or cell cultures used.
It is common practice to concentrate the virus harvest by centrifugation and to inoculate the pellet into guinea-pigs or onto solid media shown to be suitable for the detection of mycobacteria.

A.5.3.5 **Residual cell substrate DNA in cell-derived vaccines**

For viruses grown in continuous cell culture, the purified monovalent virus pool should be tested for residual cellular DNA. The purification process should be shown to consistently reduce the level and molecular size of cellular DNA (I4). This test should be appropriate for the cell culture used and should be approved by the national regulatory authority; it may be omitted with the agreement of the national regulatory authority if the manufacturing process is validated for residual DNA.

A.5.3.6 **Tests for chemicals used in production**

The concentration of each chemical added during production should be determined in the monovalent virus pool vaccine using methods approved by the national regulatory authority. The concentrations should not exceed the limits specified by the national regulatory authority. For preservatives, the national regulatory authority should approve the method of testing and the concentration.

Alternatively, tests for chemicals may be performed on the final bulk.

A.5.4 **Control of final bulk**

Final bulks are prepared by mixing and diluting monovalent virus pools of the relevant strains. In the preparation of the final bulk, only preservatives, stabilizers or other substances, including diluents, approved by the national regulatory authority should be added. Such substances should have been shown by appropriate tests not to impair the safety or effectiveness of the product in the concentrations used and should not be added before samples have been taken for any tests that would be affected by their presence. The operations necessary for preparing the final bulk should be conducted in such a manner as to avoid contamination of the product.

A.5.4.1 **Infectivity (potency)**

The influenza virus content of the clarified final bulk suspension should be determined by infectivity titration in vaccine-quality embryonated eggs or cell cultures. The number of infectious doses of vaccine should be determined (total and for each component virus) and the results expressed as EID$_{50}$, TCID$_{50}$ or pfu per human dose (see section A.7.3).

Vaccines for use during pandemics are likely to contain only one strain. Additional methods dependent on the replication of virus in
vaccine-quality embryonated eggs or cell cultures (e.g. fluorescent focus assay expressed as fluorescent focus units) may be considered for determining potency.

This test may be omitted if such a test is performed on each final lot.

A.5.4.2 Sterility

Each final bulk should be tested for sterility by a method approved by the national regulatory authority and national control laboratory.

Many countries have regulations governing sterility testing. Where these do not exist, the WHO General requirements for the sterility of biological substances (52, 58) should be satisfied.

If a preservative, stabilizer or other substance has been added to the vaccine, appropriate measures should be taken to prevent it from interfering with the sterility test.

A.5.4.3 Protein and residual DNA content

Measurements of protein and residual DNA may be specified, depending on the nature of the vaccine being produced (14, 61, 62). For example, a vaccine could be prepared from allantoic fluid and not be further purified before preparation of the final vaccine; this preparation would have an extremely high protein and ovalbumin content. Alternatively, a vaccine could be prepared from the harvested medium used to propagate cell cultures and purified to remove residual DNA and protein. The aim should be to establish parameters suitable for the production and intended use of a vaccine to ensure consistency of the composition and clinical performance of the vaccine. The parameters specified should be approved by the national regulatory authority.

A.6 Filling and containers

The requirements concerning filling and containers given in WHO good manufacturing practices for biological products (39) should apply. Single-dose containers may be preferred. If multidose containers are used, a suitable method to protect the integrity of the product before administration should be approved by the national regulatory authority.

Care should be taken to ensure that the materials of which the components of the container system are made do not adversely affect the virus content of the vaccine under the recommended conditions of storage.

A.7 Control tests on final product

Samples should be taken from each filled final lot for the tests mentioned in this section and its subsections.
A.7.1 **Identity**
An identity test should be performed on at least one container from each final lot by a method approved by the national regulatory authority and national control laboratory.

The virus strains in the final containers should be identified by methods appropriate for the haemagglutinin and neuraminidase antigens. Phenotypic and genetic information may be useful in full specific identification of the vaccine viruses.

A.7.2 **Sterility**
Vaccine (reconstituted if lyophilized) should be tested for sterility as described in section A.5.4.2.

A.7.3 **Infectivity (potency)**
The virus content of each of at least three containers selected at random from each filled final lot should be determined individually by methods fully approved by the national regulatory authority and national control laboratory.

The determination of the infectivity per dose should be based on inoculation of at least five vaccine-quality embryonated eggs or cultures per dilution, using 10-fold dilutions.

The number of infectious doses per human dose of vaccine should be determined (total and for each component virus), and the results should be expressed in EID\textsubscript{50}, TCID\textsubscript{50} or pfu per dose.

Additional methods dependent on the replication of virus in vaccine-quality embryonated eggs or cell cultures (e.g. fluorescent focus assay expressed as fluorescent focus units) may be considered for determining potency.

The requirements for virus content per human dose should be based on clinical trials in humans that demonstrate the dose range needed to ensure the safety and effectiveness of the influenza vaccines (human, live attenuated) for intranasal administration as approved by the national regulatory authority. Re-characterization of the relation of an infectious dose to a human dose may become necessary and should be undertaken with the approval of the national regulatory authority.

A.7.4 **Endotoxin**
A test for endotoxin should be included (e.g. the *Limulus* amoebocyte lysate (LAL) test) as a test of safety and manufacturing consistency of the final lot.
A.7.5  **Residual moisture of lyophilized vaccines**

The residual moisture in a representative sample of each lyophilized lot should be determined by a method approved by the national regulatory authority and the national control laboratory.

A.7.6  **Inspection of final containers**

Each container in each final lot should be inspected visually; any container showing abnormalities, such as lack of integrity, should be discarded.

A.8  **Records**

The requirements in section 8 of WHO good manufacturing practices for biological products (39) should apply.

A.9  **Retained samples**

The requirements in section 9 of WHO good manufacturing practices for biological products (39) should apply.

A.10  **Labelling**

The requirements in section 7 of WHO good manufacturing practices for biological products (39) should apply. The label on the carton, the container or the leaflet accompanying the container should state:

- that the vaccine has been prepared from virus propagated in vaccine-quality embryonated eggs (as per the description in section A.3.2) or in cell cultures;
- the type of cell line, i.e. monkey, dog, others (if appropriate);
- the strain or strains of influenza virus present in the preparation;
- the infectivity titre per human dose of the vaccine expressed as EID$_{50}$, TCID$_{50}$ or pfu (or other similar means of determining infectivity, e.g. fluorescent focus assay expressed as fluorescent focus units);
- the volume per dose and the nominal volume of vaccine in the container available for recovery and administration;
- the seasonal influenza vaccine composition for which the vaccine is intended (3);
- the name and quantity of any antimicrobial agent in the vaccine;
- the name and concentration of any preservative added;
- the temperature recommended during storage and transport;
- the expiry date;
- any special dosing schedules (e.g. two doses for a pandemic vaccine).
A.11  Distribution and transport

The requirements in section 8 of WHO good manufacturing practices for biological products (39) should apply.

A.12  Stability

A.12.1  Stability testing

Adequate stability studies form an essential part of the development of influenza vaccines (human, live attenuated) for intranasal administration. Current guidance on evaluation of vaccine stability is provided in Guidelines on stability evaluation of vaccines (38).

The stability of the influenza vaccine (human, live attenuated) for intranasal administration, including each of the active components in the vaccine in final form and at the recommended storage temperatures for the vaccine in final form, should be demonstrated to the satisfaction of the national regulatory authority on final containers from at least three final lots.

In some countries, vaccine infectivity titre should comply with the final product specifications at the expiry date (see section A.7).

Since vaccine may be stored in monovalent form for a significant period before preparation of the final bulk vaccine, stability studies may be performed on the single harvests or on the monovalent pools as well as on the final vaccine.

Formulation of the influenza vaccine (human, live attenuated) for intranasal administration must be stable throughout its shelf-life. Acceptable limits for stability should be agreed with the national regulatory authority.

Following licensure, continued monitoring of vaccine stability is recommended to support shelf-life specifications and to refine the stability profile. Data should be provided to the national regulatory authority on an annual basis.

For influenza vaccines (human, live attenuated) for intranasal administration, thermal stability for lot release should be explored to determine whether the testing provides any value in the overall understanding of vaccine quality (safety and efficacy) and the effect of production variables. If there is no added value, thermal stability should not be required as a lot release assay (38).

When any changes are made in the production process that may affect stability of the product, the influenza vaccine (human, live attenuated) for intranasal administration produced by the new method should be shown to be stable. The national regulatory authority should be informed of and approve any changes that may affect stability of the product (38).
A.12.2 Storage conditions

Storage conditions for influenza vaccines (human, live attenuated) for intranasal administration should be fully validated and approved by the national regulatory authority (38).

A.12.3 Expiry date

The expiry date should be fixed with the approval of the national regulatory authority and should take into account experimental and clinical data on the stability of the influenza vaccine (human, live attenuated) for intranasal administration (38).

In general, the expiry date should not exceed one year from the date of issue by the manufacturer because the strains used in one year’s vaccine may not be appropriate the next year (3). The national regulatory authority may approve an expiry date for a pandemic vaccine that differs from that of a seasonal influenza vaccine.

Part B. Nonclinical evaluation of new influenza vaccines (human, live attenuated)

B.1 General remarks

Nonclinical evaluation refers to all in vivo and in vitro testing performed before and during the clinical development of vaccines (20). Preclinical testing, as a subcategory of nonclinical testing, is a prerequisite for moving a candidate vaccine from the laboratory to the clinic and includes all aspects of product characterization, proof of concept/immunogenicity studies, and safety testing in animals conducted before introducing the product into humans.

A non-exhaustive summary of typical preclinical evaluations used to prepare a new influenza vaccine (human, live attenuated) for entry into clinical studies is provided in Table 1. Some of the in vitro assessments will also form the basis for and be incorporated into the ongoing nonclinical activities of the quality control and quality assurance oversight of product release.

The yearly changes made to keep vaccines current with influenza epidemiology are not expected to require repeated nonclinical studies. Some in vitro and in vivo studies, however, may be useful for assessing significant changes in manufacturing processes or alterations of critical product characteristics. Moreover, the continuation of some nonclinical activities would be expected to be appropriate for maintaining current good manufacturing practices for influenza vaccine (human, live attenuated) for intranasal administration. Current WHO Guidelines on nonclinical evaluation of vaccines: regulatory expectations (20) describe broadly applicable principles of preclinical and nonclinical assessments...
in greater detail. Guidance that may apply to the preclinical and nonclinical assessments of influenza vaccines (human, live attenuated) for intranasal administration to be used as pandemic vaccines is also provided in Regulatory preparedness for human pandemic influenza vaccines (12).

Each candidate attenuated virus donor strain to be developed for the preparation of candidate influenza vaccines (human, live attenuated) for intranasal administration should be characterized as completely as possible to identify critical genetic and phenotypic markers, to assess viral attenuation, to assess potential virus toxicity, and to determine whether the genetic basis of attenuation prevents reversion to partial or total virulence. Early laboratory studies should determine the genetic elements responsible for the virus attenuation, singly and in the combination to be present in the distributed vaccine. It is advisable to select attenuated virus donor strains with stable markers that are not dependent on retention of the markers of attenuation. The presence of additional stable markers can be used to differentiate vaccine strains from wild-type virus reference strains or other vaccine viruses in epidemiological surveillance.

The delivery device may have an impact on the uptake of a vaccine, its safety and its effectiveness (20). Influenza vaccines (human, live attenuated) may be administered intranasally using proprietary or nonproprietary delivery devices including syringes, sprayers and other liquid delivery devices. The preclinical evaluation of influenza vaccines (human, live attenuated) should include a rigorous assessment to establish the suitability of the intranasal delivery device to support the safety and effectiveness of the vaccine.

Table 1
Nonclinical evaluation* of new influenza vaccines (human, live attenuated) for intranasal administration

<table>
<thead>
<tr>
<th>Area of nonclinical evaluation</th>
<th>Primary concern</th>
<th>Scope of nonclinical evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vitro assessments</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Process development, quality control and quality assurance</td>
<td>Process is expected to meet cGMP standards</td>
<td>Process control and laboratory studies related to all steps in process to prepare vaccine</td>
</tr>
<tr>
<td>Product characterization</td>
<td>Product quality is appropriate for use in preclinical and clinical studies</td>
<td>Genetic, biochemical, and biological characteristics of vaccine including features of attenuation</td>
</tr>
</tbody>
</table>

continues
Table 1 continued

<table>
<thead>
<tr>
<th>Area of nonclinical evaluation</th>
<th>Primary concern</th>
<th>Scope of nonclinical evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In vivo assessments</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toxicity and safety testing</td>
<td>Product risks are appropriate for anticipated uses</td>
<td>Safety indicators such as: range of safe dose; single and repeated safe doses; safety parameters for clinical monitoring; potential for reversion to virulence</td>
</tr>
<tr>
<td>Immunogenicity and efficacy</td>
<td>Product effects are appropriate for anticipated uses</td>
<td>Effect indicators such as: type of immune responses for clinical evaluation; frequency and duration of immune responses; priming and boosting parameters; protective effects in animal challenge studies</td>
</tr>
</tbody>
</table>

* Sources of guidance on nonclinical evaluation of vaccines: WHO; International Conference on Harmonisation; European Medicines Agency; United States Food and Drug Administration; Medical Research Council.

**B.2 Process development and product characterization**

Before clinical trials are initiated, it is generally advisable for production processes and the product itself to be in the expected final form. However, the information needed to proceed to clinical trials may vary with the nature of the vaccine. The preclinical and nonclinical development plans should therefore be discussed with and approved by the national regulatory authority and the national control laboratory.

Although all preclinical and most nonclinical studies will be performed before licensure for an influenza vaccine (human, live attenuated) for intranasal administration, the suitability of an attenuated virus donor strain needs continuous careful review (see section A.4.5.3.2). This review ensures that the candidate influenza vaccine viruses containing prevalent antigens from new wild-type virus reference strains do not substantially alter the established parameters of safety and effectiveness confirmed by clinical studies. The development and use of influenza vaccines (human, live attenuated) for intranasal administration therefore require long-term commitment by manufacturers and national regulatory authorities to conduct laboratory studies as part of the nonclinical evaluation of the vaccines.

Attenuation of influenza vaccines (human, live attenuated) for intranasal administration is based on the attenuated virus donor strain used for vaccine
preparation (see section A.4.5.3.2). A complete genetic sequence of the attenuated virus donor strain should be obtained in order to map as carefully as possible where attenuating mutations have been introduced. Since each passage of an influenza virus may introduce new mutations, the possibility exists for reversion to a less attenuated or fully virulent form of virus. Studies should therefore be carried out to determine whether reverting mutations appear at any point in the planned production and use of vaccine strains. These studies should also determine, to the extent possible, whether the haemagglutinins and neuraminidases of the wild-type influenza viruses affect the stability of attenuating mutations.

For example, when a highly pathogenic avian influenza virus is used, close attention should be paid to ensuring that the haemagglutinin retains the modifications that eliminate the highly pathogenic phenotype.

Phenotypic markers should be mapped genetically to the extent possible with in vitro systems. Key phenotypic features and adaptations of the attenuated virus donor strain should be demonstrated. For example, the ability to replicate efficiently at relatively low temperature should be demonstrated consistently for a cold-adapted attenuated virus donor strain. For temperature-sensitive attenuated virus donor strains, it should be possible to demonstrate reproducibly the temperature at which viral replication is inhibited. In vitro studies should be confirmed for the candidate influenza vaccine (human, live attenuated) seed viruses in order to ensure that attenuating features are fully retained throughout the vaccine process steps.

B.3 Nonclinical toxicity and safety testing

B.3.1 Preclinical toxicity

Preclinical toxicity studies are designed primarily to demonstrate the safety and tolerability of a candidate influenza vaccine (human, live attenuated) for intranasal administration. The design of a preclinical toxicity study should meet the criteria outlined in a specific, written protocol in order to support an intended clinical trial. The protocol should state the background, rationale and objectives of the nonclinical studies and describe the design, methodology and organization, including statistical considerations, and the conditions under which studies are to be performed and managed. WHO has published Guidelines on nonclinical evaluation of vaccines: regulatory expectations (20).

Toxicity tests for influenza vaccines (human, live attenuated) for intranasal administration should include:

- an evaluation of the initial safe dose and of subsequent dose escalation schemes relevant to the clinical dose;
- an evaluation of single and repeated doses as appropriate;
- a determination of a set of relevant safety parameters for clinical monitoring;
- a demonstration of potential reversibility of virulence of attenuated vaccine strains; and
- local tolerability studies, which are typically included as part of the general toxicity evaluation.

The toxicity assessment of formulations of influenza vaccines (human, live attenuated) for intranasal administration can be done either in stand-alone animal toxicity studies or in combination with studies of safety and activity that include toxicity end-points in their design (20). The parameters to be considered in designing animal toxicity studies with influenza vaccines (human, live attenuated) for intranasal administration include:

- relevant animal species, e.g. ferrets, mice, non-human primates;
- ability to infect the animal, including sero-susceptibility status;
- virus strain, i.e. seasonal influenza strains, novel human influenza viruses, pandemic viruses;
- dosing schedule, i.e. one, two or three doses;
- method of vaccine administration, e.g. nasal spray or nasal drops;
- timing of evaluation of end-points; and
- clinical chemistry, antibody responses, histological examination of target organs, and necropsy.

Despite efforts to maximize the predictive value of nonclinical toxicity studies, animal models have limited applicability to human experiences and it is possible that the animal studies will not accurately reflect the risks of using influenza vaccines (human, live attenuated) for intranasal administration in humans.

The design and value of repeated-dose toxicity tests should be considered on a case-by-case basis. If an influenza vaccine (human, live attenuated) for intranasal administration is intended to be clinically tested in women of childbearing age, the need for reproductive toxicity studies and studies of embryo-fetal and perinatal toxicity should be considered on a case-by-case basis. Reproductive toxicity studies, where appropriate, will need to be undertaken before licensing (20).

Any changes in the composition of the human influenza vaccine (live, attenuated) for intranasal administration should be carefully considered both by the manufacturer and by the national regulatory authority. In general, there should be no need to repeat the toxicity assessments for an annual strain
change as long as attenuation has been adequately documented. However, it may be necessary to repeat some or all of the toxicity studies when a novel human influenza virus subtype emerges (e.g. influenza A virus H5N1) and a new influenza vaccine (human, live attenuated) for intranasal administration is prepared. Special considerations on the preclinical and nonclinical evaluation of influenza vaccines (human, live attenuated) for intranasal administration for pandemic use are provided in Regulatory preparedness for pandemic influenza vaccines (12).

Any changes in formulation, ingredients or excipients in the human influenza vaccine (live, attenuated) for intranasal administration may require some or all of the toxicity studies to be repeated. The manufacturer should discuss these changes with and secure the approval of the national regulatory authority.

B.3.2 Preclinical safety testing

Every effort should be made in the preclinical studies to identify markers of attenuation (genetic sequences) that can be used to monitor the results during clinical evaluation phases. Primary investigations of attenuation in animals may be done in a number of influenza-responsive species such as ferrets, mice, and non-human primates to establish the relationship of specific genetic features to the attenuation phenotype. Studies in animals may be designed to confirm the location and degree of replication of candidate influenza vaccine (human, live attenuated) in the respiratory tract of the host.

Results from animals inoculated with the attenuated virus donor strain (or its derivative candidate vaccine virus) compared with results from animals inoculated with the wild-type reference virus strain should indicate whether the attenuated virus donor strain (or the candidate vaccine virus) is adequately safe to allow limited clinical trials to be undertaken.

The potential of influenza vaccines (human, live attenuated) for intranasal administration for neuro-invasiveness and neuro-virulence should be considered if the wild-type virus used for seed virus preparation demonstrates neuro-invasiveness. The national regulatory authority should be consulted on the need for preclinical evaluation of the potential for neurological effects in suitable animal models.

Animal studies cannot completely predict what will happen in humans because of differences in anatomy, host temperature, and other variables. Features that appear to be attenuating in animals may not fully predict the genotype or phenotype of attenuation in humans. In addition, the vaccine dose tolerated by animals may differ greatly from that tolerated by humans. Results of studies in animals therefore do not obviate the need for confirmation in clinical trials in humans.
B.4 Nonclinical immunogenicity and efficacy

B.4.1 Nonclinical immunogenicity

Assessment of immune responses in animals can provide some assurance that an influenza vaccine (human, live attenuated) has replicated in the host. Influenza virus infection triggers a multitude of immune responses in the innate and adaptive immune systems, which can be assessed in an animal model. Although there is no specific correlate of protection defined for influenza vaccine (human, live attenuated) for intranasal administration, antibodies directed against viral haemagglutinins in the blood and mucosal secretions have been shown to have protective effects in animal models and in humans. Antibody responses to neuraminidase and other viral proteins, as well as cellular responses involving T helper and cytotoxic cells, have also been identified during recovery from influenza infection. Despite the lack of specific correlates of protection, immune responses in animals should be evaluated for their potential to provide an in vivo correlation with prevention of infection and/or reduction of disease signs and pathogenicity.

The specific immune parameters to assess in animals depend, in part, on the nature of the influenza vaccine (human, live attenuated) for intranasal administration. Parameters should at least include antibodies directed against haemagglutinins (e.g. serum neutralizing, haemagglutination inhibition, or single radial haemolysis antibodies).

While immune responses in animals do not necessarily predict immune responses in humans, it is nevertheless possible to derive useful information from preclinical immunogenicity studies.

B.4.2 Nonclinical efficacy

Protective efficacy can be assessed directly after immunization in animals by infectious challenge with wild-type or selected laboratory strains of influenza viruses. Preclinical studies in animals also allow assessment of the protective effects of a candidate influenza vaccine (human, live attenuated) for intranasal administration against potentially lethal influenza virus infections, which may become important in preparing vaccines for highly pathogenic influenza viruses with pandemic potential. Furthermore, animal studies can be designed to assess the breadth of immune and clinical protection of a given influenza vaccine (human, live attenuated) for intranasal administration against different influenza A subtypes and drift variants that may indicate the need for new antigens in the vaccine.

While efficacy identified in an animal model does not necessarily predict the protective effect in humans, it is nevertheless possible to derive useful information from preclinical efficacy studies in animals.
Part C. Clinical evaluation of new influenza vaccines (human, live attenuated)

C.1 General remarks

The clinical evaluation of new influenza vaccines (human, live attenuated) for intranasal administration includes studies undertaken as part of the developmental process, the licensure procedure, and/or studies performed in the post-marketing period. Extensive guidance for manufacturers and regulatory authorities on the clinical development of vaccines is found in the WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (18) and Guidelines for good clinical practices (GCP) for trials on pharmaceutical products (63). Guidance that may apply to the clinical evaluation of influenza vaccines (human, live attenuated) for intranasal administration to be used as pandemic vaccines is provided in Regulatory preparedness for human pandemic influenza vaccines (12).

There are a number of issues to be considered in the clinical development and use of safe and effective influenza vaccines (human, live attenuated) for intranasal administration. Since influenza vaccines generally have been multivalent to provide protection against influenza A and influenza B viruses, each component of influenza vaccines (human, live attenuated) for intranasal administration must be proved safe and protective in the context of a formulated multivalent vaccine (64). Since wild-type influenza viruses constantly mutate, influenza vaccines (human, live attenuated) for intranasal administration will require frequent re-evaluations of composition and change, as recommended by WHO, on the influenza viruses to use in vaccines in countries of the northern and southern hemispheres (3).

There is no established immune correlate of protection for influenza vaccines (human, live attenuated) for intranasal administration and it is possible that immune correlates may be specific for individual influenza vaccines (human, live attenuated) (63). Despite potential difficulties, large field trials and routine immunization campaigns have shown that satisfactory influenza vaccines (human, live attenuated) for intranasal administration can be prepared, distributed and administered (8, 27, 64).

Possible variation in the attenuating features of each influenza vaccine (human, live attenuated) for intranasal administration make it important to carefully characterize the vaccine viruses derived from a new attenuated donor virus before licensure. Although animal studies may not perfectly predict human clinical experiences, nonclinical and preclinical studies with an influenza vaccine (human, live attenuated) for intranasal administration should help in determining the genetic elements of attenuation in humans, evaluating the possibility of reversion to partial or full virulence post-vaccine administration, and in determining potential targets for safety data collection as the clinical trials begin.
The delivery device may have an impact on the uptake of a vaccine, its safety and its effectiveness (20). Influenza vaccines (human, live attenuated) may be administered intranasally using proprietary or nonproprietary delivery devices – syringes, sprayers and other liquid delivery devices. The clinical evaluation of influenza vaccines (human, live attenuated) should include a rigorous assessment of the suitability of the intranasal delivery device to support the safety and effectiveness of the vaccine.

C.2 Clinical evaluation strategy
The clinical development of a new influenza vaccine (human, live attenuated) for intranasal administration will resemble that of other vaccine products; a typical strategy is provided in Table 2.

C.2.1 Clinical studies for licensure
Early (Phase I) studies focus on safety aspects, including replication, shedding and transmission potential of the vaccine virus, relevant pharmacokinetics, and neurotoxic and immunological effects identified in the nonclinical evaluation of the vaccine virus. Early clinical studies (Phase I and Phase II) should be designed to confirm the dose range that is well tolerated. These studies are recommended because replication of influenza vaccine (human, live attenuated) may be affected by previous host exposure to influenza viruses or vaccines as well as by underlying host factors.

As studies progress (Phase II), more information is acquired on immunological and protective effects, and trials to confirm efficacy (usually Phase III) are also undertaken. Later clinical studies (Phase II and Phase III) should be done to expand the number of subjects to be evaluated by groups targeted for vaccine use and to define the types and frequency of adverse events that have already been identified. Later clinical studies should also make it possible to identify rarer events that can only be detected when a sufficiently large population has been examined.

Table 2
Clinical evaluationa of new influenza vaccines (human, live attenuated) for intranasal administration

<table>
<thead>
<tr>
<th>Phases of clinical evaluation</th>
<th>Primary concern</th>
<th>Scope of clinical evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase I</td>
<td>Safety (for common events)</td>
<td>Small-scale trials to determine whether significant risks exist</td>
</tr>
</tbody>
</table>

continues
Phases of clinical evaluation | Primary concern | Scope of clinical evaluation
--- | --- | ---
Phase II | Preliminary effect (immunogenicity) and general safety (for typical and less common events) | Larger-scale trials to further define risks and potential benefits in populations expected to use the vaccine
Phase III | Protective efficacy (pivotal) and safety (for uncommon events) | Trial size defined to provide statistical certainty for specific end-points related to safety and efficacy
Post-marketing clinical trials | Safety (for rare events) and effectiveness (to expand on original observations) | Trials specifically designed to further refine information on safety and efficacy in larger populations and/or in new populations for vaccine use
Post-marketing surveillance | Safety (for unexpected and rare events and/or signals) | Potentially includes all vaccine recipients to identify safety signals arising from routine use and expanded population studies

Later clinical studies also assess the potential for variation among people of different ages. People with pulmonary, cardiac or immune dysfunction should be addressed as part of expanding vaccine studies. It is also desirable to establish immune correlates of protection in human populations, since this may aid future improvements in the production and evaluation of influenza vaccine (human, live attenuated) for intranasal administration.

The information needed for the successful completion of clinical trials may vary somewhat depending on the nature of the vaccine; clinical development plans should therefore be discussed with and approved by the national regulatory authority and the national control laboratory. Current WHO guidelines on clinical evaluation of vaccines describe broadly applicable principles in greater detail (18). Guidance that may apply to the clinical assessments of influenza
vaccines (human, live attenuated) for intranasal administration to be used as pandemic vaccines is provided in Regulatory preparedness for human pandemic influenza vaccines (12) and Guidelines on clinical evaluation of vaccines: regulatory expectations (18).

C.2.2  Post-marketing studies

Although most of the clinical development will precede licensure of influenza vaccines (human, live attenuated) for intranasal administration, post-marketing trials and surveillance will provide significant information about the safety and effectiveness of vaccines that cannot be predicted or detected in the pre-licensing period, particularly with regard to rare adverse events. Manufacturers and national regulatory authorities should therefore make a substantial commitment to pharmacovigilance and to clinical studies as part of a continuing, post-marketing review of influenza vaccines (human, live attenuated) for intranasal administration. In the case of a significant change in the manufacturing process, the national regulatory authority may also require clinical studies.

While clinical studies with novel influenza virus subtypes or lineages may be considered, once adequate experience with multiple candidate influenza vaccine viruses within a subtype or lineage has accumulated, post-marketing clinical trials may no longer be justified for WHO-recommended changes to the influenza viruses to be used in vaccines in countries of the northern and southern hemispheres (3).

Some national regulatory authorities may require a limited clinical evaluation to assess safety parameters for licensing purposes whenever a new candidate influenza vaccine virus is introduced.

C.3  Clinical safety

C.3.1  Initial safety assessment

An influenza vaccine (human, live attenuated) is usually given intranasally so that virus replicates in the respiratory tract tissues, predominantly the nasopharyngeal mucosa. As a result of replication of influenza vaccines (human, live attenuated) viruses in the respiratory tract, mild upper respiratory symptoms and occasional benign systemic reactions have been observed in some individuals, but with much lower frequency and severity than with wild-type influenza viruses. Initial vaccine safety trials should include a careful assessment of symptoms related to influenza virus infection.

Since vaccinated individuals may shed influenza vaccine (human, live attenuated) viruses via respiratory secretions, virus excretion should be assessed for quantity and duration, and, if possible, compared with shedding during infection with relevant virulent wild-type influenza viruses. The excreted attenuated
virus strains should maintain the characteristic phenotypes of influenza vaccine (human, live attenuated) viruses, which may be partially predicted during the nonclinical and preclinical vaccine evaluation.

Initial clinical studies (Phase I), particularly with newly developed influenza A subtypes, are best performed in an environment offering the maximum guarantee of isolation and discipline. Some guidance on this may be found in Biosafety risk assessment and guidelines for the production and quality control of human influenza pandemic vaccines (13). These clinical trials are meant to ensure that the virus is not excreted at an unacceptably high titre or for an excessively long period. Clinical trials should show that the frequency of virus transmission is consistent with public health goals.

In addition to detailed assessment of safety, early clinical trials should attempt to show that the benefit–risk ratio of vaccine use is acceptable.

C.3.2 Expanded safety assessment

When determining the acceptability of an influenza vaccine (human, live attenuated) for intranasal administration in larger-scale clinical trials, limited studies in which the vaccine is administered to immunologically primed individuals from different age groups may be undertaken before the vaccine is administered to immunologically naïve individuals. The follow-up period for the assessment of safety should take into account effects that may occur many days or weeks post-administration. It should also include purposeful review of events for at least six months after final vaccine administration for a trial protocol for both early and later trials examining vaccine safety. Guidance on age progression (from adult to younger age groups) for an influenza vaccine (human, live attenuated) for intranasal administration in children has been published by WHO (12, 63).

Phase II and Phase III clinical trials should delineate the type, frequency and severity of local and systemic respiratory infection-related events such as sneezing, nasal discharge, cough, sore throat, fever and myalgia, as well as other respiratory and non-respiratory events that have been observed in preclinical studies and Phase I clinical trials. As numbers expand during Phase II and Phase III studies, and post-marketing studies, rarer adverse events may be identified for further attention.

Each influenza virus passage may introduce new mutations. Studies should therefore be pursued to determine whether reverting mutations appear at any point in the planned production and use of new attenuated donor viruses.

The possibility exists of influenza vaccine (human, live attenuated) virus transmission through contact of susceptible individuals with virus-containing secretions. However, the probability of such transmission should be small compared with that of wild-type influenza viruses, even in immunologically naïve people.

When novel wild-type reference virus strains (e.g. in the case of a pandemic vaccine) are used in the preparation of influenza vaccines (human,
live attenuated), clinical studies may be considered as a part of the assessment to determine that the newly introduced haemagglutinin and neuraminidases have not altered the safety profile of the influenza vaccine (human, live attenuated) for intranasal administration in any adverse manner.

C.4 Clinical efficacy and immunogenicity

With current methods and capabilities, clinical end-point studies provide the definitive assessment of efficacy of influenza vaccines (human, live attenuated) for intranasal administration (64). Several antibody responses, including neutralizing antibodies in blood and respiratory secretions, are associated with prevention of influenza virus infection as well as reduction of symptoms and overall pathological effects in humans. However, broadly applicable immunological correlates of protection have not been identified and relevant immune parameters may vary for influenza vaccines (human, live attenuated) for intranasal administration (63). Nonetheless, opportunities to correlate specific immune parameters with prevention of infection or illness should not be missed during clinical studies.

C.4.1 Clinical efficacy studies

Vaccine efficacy is the reduction in the odds of developing clinical disease post-vaccination relative to the odds when unvaccinated. Efficacy of influenza vaccines (human, live attenuated) for intranasal administration in a specified population should be demonstrated in adequately-powered, well-controlled clinical trials (Phase III). Since this is usually a complex and expensive undertaking and requires an infected population, the design and feasibility of efficacy clinical trials (e.g. of pandemic vaccines) should be thoroughly explored with the competent authority (12, 18).

In general, efficacy studies should demonstrate that influenza vaccines (human, live attenuated) for intranasal administration prevent laboratory (e.g. culture) confirmed influenza illnesses. The ability to document vaccine efficacy depends upon an adequate attack rate of influenza (proportion of population exposed to influenza viruses who become clinically ill) in relation to the ability to prevent infection in a study with an appropriate sample size. Since attack rates for influenza vary from year to year and different influenza types or subtypes predominate in different years, efficacy studies will generally require a large enrolment and sometimes a multi-year plan for thorough evaluation of each component strain, type or subtype contained in influenza vaccines (human, live attenuated) for intranasal administration.

C.4.2 Clinical correlates of protection

If possible, clinical trials should establish a correlate of protection that can be used to guide development of influenza vaccines (human, live attenuated) for intranasal
administration. Since influenza vaccines (human, live attenuated) are administered by the respiratory route, it should be anticipated that immune parameters other than antibodies in blood could serve as correlates of protection (63). Novel methods to measure antibody or cellular responses should be validated.

C.4.3 Clinical serological parameters

Anti-haemagglutinin antibodies in blood are the most widely studied immune mechanisms in humans. However, antibody responses to neuraminidase and other influenza viral proteins may also contribute to protection. The specific serological parameters to assess in clinical trials (Phases I–III) depend in part on the nature of the influenza vaccine (human, live attenuated) for intranasal administration but a reasonable starting point is determination of neutralizing, haemagglutination inhibition or single-radial haemolysis antibodies (63).

C.4.4 Other clinical measures of immunity

Assessment of innate, mucosal and cellular immune mechanisms may be helpful in further developing a rationale for protection resulting from influenza vaccines (human, live attenuated) for intranasal administration.

Influenza vaccines (human, live attenuated) for intranasal administration are expected to induce local innate immune responses, including interferon and other cytokines, as well as adaptive immune responses such as secretory mucosal antibodies, i.e. IgA (8, 63).

Cellular immune mechanisms are less well understood but it is expected that influenza vaccines (human, live attenuated) for intranasal administration may also mimic the T-cell responses identified during naturally occurring infections in humans.

C.5 Evaluation in special populations

In general, clinical trials should be performed first in the least vulnerable population (i.e. healthy adults). Placebo groups are essential to evaluating the incidence and intensity of adverse events following immunization.

If the initial clinical trials in healthy adults demonstrate vaccine safety at specific doses, additional studies may be undertaken to delineate the uses of influenza vaccines (human, live attenuated) for intranasal administration in different age and risk groups. Because it may be inappropriate to expose some vulnerable populations to an influenza vaccine (human, live attenuated) for intranasal administration, early clinical studies should also provide an understanding of the frequency of transmission and the potential for adverse consequences of transmission to non-vaccinated individuals.
C.5.1  **Children**

If clinical trials in adults demonstrate a safety profile that is suitably benign, studies in children may be undertaken to evaluate safety and efficacy of influenza vaccines (human, live attenuated) for intranasal administration (12, 63). It may be prudent to study older children first and then progressively younger children: younger children would be expected to be more prone to develop local and systemic adverse events that may not be apparent in older individuals who have been exposed to wild-type influenza viruses and vaccines.

C.5.2  **The elderly**

If clinical trials in healthy adults demonstrate a safety profile that is suitably benign, studies in elderly populations, particularly those with chronic diseases, may be undertaken to evaluate the safety and efficacy of influenza vaccines (human, live attenuated) for intranasal administration. Although there may be similarities between healthy adults and the elderly, different responses to influenza vaccines (human, live attenuated) for intranasal administration may result from lifetime exposure to influenza and from the accumulation of chronic, particularly cardiac and pulmonary, illnesses in the elderly.

C.5.3  **Risk groups**

A solid understanding of the typical post-vaccination clinical course in the healthy host is imperative before any study is initiated in a population with special risk status. All studies should be conducted with strict ethical considerations for the welfare of participants and no one should be exposed to unreasonable risk (18, 63).

Special studies may be considered for populations for whom influenza vaccination is especially important (such as subjects with chronic bronchitis and asthma) but in whom potential adverse events might be more severe.

People with immune defects that do not contraindicate exposure to a live virus vaccine should be evaluated cautiously to determine whether an influenza vaccine (human, live attenuated) for intranasal administration is beneficial without any unusual consequences such as prolonged shedding of virus.

C.6  **Strain change considerations for seasonal vaccines**

After an initial licensure, the vaccine is reformulated according to the circulating strains predicted for the upcoming season (3). For inactivated influenza vaccines, small-scale immunogenicity studies are requested in some parts of the world. However, without immunological correlates of protection, such studies with influenza vaccines (human, live attenuated) for intranasal administration are of limited value. Moreover, an annual clinical efficacy trial is generally not feasible because of methodological constraints and timing.
Some national regulatory authorities may recommend small-scale safety studies for influenza vaccines (human, live attenuated) for intranasal administration. The value of such studies for reassessing the benefit–risk balance is unknown. In general, when the vaccine production process remains unchanged, it is unlikely that the benefit–risk balance as assessed at the time of licensure will change. The need for additional clinical evaluations before annual re-licensure may depend upon factors that include major changes in subtypes (i.e. antigenic drifts or shifts), previously identified risks and/or reported safety signals, and impaired efficacy during vaccine use in the previous season. In such cases, a clinical study to reconfirm the benefit–risk balance may be deemed necessary.

It follows that a quality assessment of vaccine formulation for the upcoming year may be recommended and may be supplemented with a structured annual post-marketing surveillance programme in countries where vaccine is used in wide-scale vaccination programmes. Such post-marketing surveillance programmes may change over time, depending on experience. In the initial years following licensure, post-marketing surveillance studies may be requested and be replaced later by a programme of continuous surveillance. In any case, marketing authorization holders are recommended to consult with national regulatory authorities where vaccine is to be marketed and discuss details of the post-marketing surveillance programme.

C.7 Vaccines intended for pandemic influenza

It is expected that attenuated virus donor strains developed for seasonal influenza vaccines (human, live attenuated) for intranasal administration may be evaluated for use in pandemic situations. New attenuated virus donor strains may be prepared specifically for pandemic use. If a new attenuated virus donor strain is planned for pandemic use, the quality, safety and efficacy of pandemic influenza vaccines (human, live attenuated) for intranasal administration should be ensured by conformity with the recommendations in the present document. In addition, special regulatory considerations for pandemic influenza vaccines should be followed (12).

For an attenuated donor strain specifically prepared for pandemic use, studies should demonstrate an attenuated phenotype and stability of the attenuated donor strain genome. Clinical trials of influenza vaccines (human, live attenuated) for intranasal administration containing the haemagglutinin and neuraminidase of novel influenza viruses should be conducted in isolation units while the novel virus with pandemic potential is not spreading efficiently from human to human (i.e. WHO pandemic phases 1–5). Isolation procedures should be maintained for recipients of vaccines against novel influenza viruses for the duration of vaccine virus shedding. WHO will provide advice to national health authorities on declaration of pandemic phases. Assessment of efficacy
in the absence of widespread disease is especially challenging. Given the potential efficacy of influenza vaccines (human, live attenuated) for intranasal administration in paediatric populations, approval for the conduct of studies in children will be needed from national regulatory authorities.

Comprehensive guidance on influenza vaccines (human, live attenuated) for intranasal administration to be used as pandemic vaccines is provided in Regulatory preparedness for human pandemic influenza vaccines (12).

**Part D. Recommendations for national regulatory authorities**

**D.1 General remarks**

The general recommendations for national regulatory authorities contained in WHO good manufacturing practices for biological products (39) should apply. An extensive list of WHO guidelines with additional specific recommendations for the quality assurance of biological products to be followed by national regulatory authorities is provided in the reference section of this document.

The national regulatory authority should direct manufacturers on the influenza virus strains to be used and the recommended human doses.

The national regulatory authority should take into consideration all information available on strains before deciding on those permitted for vaccine production (3).

In addition, the national regulatory authority should provide a reference preparation of influenza vaccines (human, live attenuated) for intranasal administration to check the normal susceptibility of the titration system. The national regulatory authority should also specify the virus content requirements to be fulfilled in order to achieve adequate immunization with the recommended human dose.

As a practical matter, the national regulatory authority will need to collaborate with the manufacturer to develop an acceptable reference preparation.

**D.2 Release and certification**

A vaccine lot should be released only if it fulfils national requirements and/or Part A of these recommendations. A protocol based on the model in Appendix 1, signed by the responsible official of the manufacturing establishment, should be prepared and submitted to the national regulatory authority in support of the request for release of vaccine for use. A statement signed by the authorized
official of the national regulatory authority should be provided at the request of
the manufacturing establishment, certifying that the lot of vaccine in question
meets all national requirements as well as the manufacturing recommendations
provided in Part A of these recommendations.

The release certificate should state the number under which the lot was
released by the national regulatory authority and the number appearing on the
labels of the containers. Importers of influenza vaccines (human, live attenuated)
for intranasal administration should be given a copy of the official national
release document of the country of production. The purpose of the certificate is
to facilitate the exchange between countries of influenza vaccines (human, live
attenuated) for intranasal administration. An example of a suitable certificate is
given in Appendix 3.

D.3 Manufacturing changes

In the case of a new vaccine, the national regulatory authority should assess
vaccine safety and efficacy by arranging for studies in human volunteers of one
or more of the lots of influenza vaccine (human, live attenuated) for intranasal
administration that have satisfied the above-mentioned recommendations. Such
studies should include assessment of the immune responses and adverse reactions
in various age groups.

In the case of significant change in the manufacturing process, preclinical,
nonclinical and clinical studies may also be required by the national
regulatory authority.

Some national regulatory authorities require a limited clinical evaluation
for licensing purposes whenever a new vaccine strain is introduced.

Authors

The first draft of these revised WHO Recommendations for the production
and control of influenza vaccine (human, live attenuated) was prepared in May
2008 by Dr C.P. Alfonso, WHO, Geneva, Switzerland, and Dr R. Levandowski,
Bethesda, MD, USA.

Comments on the first draft were provided by Dr E. Govorkova,
Department of Infectious Diseases, St Jude Children’s Research Hospital,
Memphis, TN, USA; Dr M. Pfleiderer, Paul-Ehrlich-Institute, Langen, Germany;
and Dr J. Weir, Center for Biologics Evaluation and Research, Food and Drug
Administration, Rockville, MD, USA.

A draft incorporating these comments was produced in December
2008 and further revised to generate a document that was reviewed at the
informal consultation on WHO Recommendations for the production and
control of influenza vaccine (human, live attenuated), 26–27 February 2009, Geneva, Switzerland. The informal consultation was attended by stakeholders from national immunization programmes, basic research and public health sciences, regulatory authorities, and vaccine manufacturers. The following were members of the drafting group and participated in the informal consultation: Dr K. Edwards, Pediatric Clinical Research Office, Vanderbilt University School of Medicine, Nashville, TN, USA; Dr R. Levandowski, Bethesda, MD, USA; Mrs T. Marengo, Nesles-la-Vallée, France; Professor A. Osterhaus, Department of Virology, Erasmus MC, Rotterdam, the Netherlands; Dr M. Pfleiderer, Viral Vaccines, Paul-Ehrlich-Institute, Langen, Germany; Dr H. Van de Donk, The Hague, the Netherlands; Dr B. Voordouw, Pijnacker, the Netherlands; Dr J. Weir, Division of Viral Products, Center for Biologics Evaluation and Research, Food and Drug Administration, Rockville, MD, USA. The following stakeholders also participated in the informal consultation: Dr B. Breedveld, Global Regulatory Affairs, Schering-Plough, Oss, the Netherlands; Dr R.A. Bright, Influenza Vaccine Project Vaccine Development Global Program, Program for Appropriate Technology in Health (PATH), Washington, DC, USA; Dr F. Cano, Unité Contrôle des Vaccins Viraux et Sécurité Virale, AFSSAPS Direction des Laboratoires et des Contrôles, Lyons, France; Dr K. Coelingh, MedImmune Inc., Seattle, WA, USA; Dr T. Colegate, Novartis Vaccines, Liverpool, England; Dr R. Dhere, Vaccine Production, Serum Institute of India Ltd., Pune, India; Dr N. Hidayati, Sub-Directorate of New Drug Evaluation, Directorate of Drug and Biological Product Evaluation, National Agency of Drug and Food Control, Jakarta Pusat, Indonesia; Dr I. Krasilnikov, Research and Development, "Microgen" State Company, Moscow, Russian Federation; Mrs Kusmiaty, Biological Products Division, National Quality Control Laboratory of Drug and Food Institution, National Agency of Drug and Food Control, Jakarta Pusat, Indonesia; Mrs P. S. Thanaphollert, Biological Products Section, Drug Control Division, Food and Drug Administration, Ministry of Public Health, Nonthaburi, Thailand; Dr A. Mironov, Clinical Trials Department, “Microgen” State Company, Moscow, Russian Federation; Professor L.G. Rudenko, Department of Virology, Russian Academy of Medical Science, Institute for Experimental Medicine, St Petersburg, Russian Federation; Mrs P.S. Thanaphollert, Biological Products Section, Drug Control Division, Food and Drug Administration, Ministry of Public Health, Nonthaburi, Thailand; Dr H. van den Bosch, Nobilon, Boxmeer, the Netherlands; Professor Le van Phung, National Institute for Control of Vaccine and Biologicals, Hanoi City, Viet Nam; Dr R. Walker, Vice-President, Medical and Scientific Affairs, MedImmune Inc., Gaithersburg, MD, USA; Dr J. Wood, Division of Virology, National Institute for Biological Standards and Control, Potters Bar, England. The informal consultation was also attended by the following WHO staff: Dr C.P. Alfonso, Quality, Safety and Standards, WHO, Geneva, Switzerland; Dr P. Ghimire,
In June 2009, a revised draft was produced to reflect comments and input received from stakeholders from national immunization programmes, basic research and public health sciences, regulatory authorities, and vaccine manufacturers during the informal consultation of February 2009. The revised draft was sent to these stakeholders for review and submission of additional comments by mid-July 2009.

A further revision was produced in the light of comments received and was proposed for endorsement by the Expert Committee on Biological Standardization at its meeting in October 2009 in Geneva, Switzerland.

The present recommendations reflect the discussions of the Expert Committee on Biological Standardization; with requests for certain modifications, the Expert Committee endorsed the document.

Acknowledgements

Acknowledgments are due to Dr Roland Levandowski for his expert advice and for having laid the foundation of these recommendations.

Acknowledgements are also due to Mrs Christine Jolly and Mrs Diana de Toth for their secretarial assistance and programme support at WHO Headquarters.

References


Appendix 1

Summary protocol for influenza vaccines (human, live attenuated) for intranasal administration (master/working seed lot Type A or Type B)

The model summary protocol that follows is provided as general guidance to manufacturers. It is not intended to constrain them in the presentation of data relevant to the complete review of the quality control tests performed on the vaccine. It is important to note that satisfactory test results do not necessarily imply that the vaccine is safe or effective, since many other factors must be taken into account, including the characteristics of the manufacturing facilities.

Name and address of manufacturer ____________________________
Laboratory reference no. of lot ____________________________
No. of lyophilized lot (if applicable) __________________________
Date when the processing was completed ____________________

Information on manufacture

Substrate for manufacture

Vaccine-quality embryonated eggs or cell culture __________________________

For vaccine-quality embryonated eggs, qualification of eggs:

Layer flock status, e.g. SPF-SAN __________________________
Supplier name and address __________________________
Remarks __________________________

For cell culture, qualification of cells:

Cell bank reference no. __________________________
Species and tissue origin __________________________
Passage history __________________________
Remarks __________________________

Virus used to inoculate vaccine-quality embryonated eggs or cells for the manufacture of the lot:

Strain and sub-strain __________________________
Passage level __________________________
Source and reference no. __________________________
Remarks __________________________
Results of sterility test __________________________
Results of tests for adventitious agents ______________________________________
Results of tests for attenuation ____________________________________________
Conditions of storage ____________________________________________________

**Monovalent virus pool Type A or Type B**

Name and address of manufacturer __________________________________________
Laboratory reference no. of the virus pool ____________________________________

*Virus used to inoculate vaccine-quality embryonated eggs or cells*
  - Master seed strain and source ___________________________________________
  - Passage level of master seed ____________________________________________
  - Working seed lot, reference no. and source ________________________________

**Date of inoculation**
  - Date of harvesting allantoic or amniotic fluid or cell-culture fluids __________
  - Storage conditions before use in final bulk _________________________________
  - Clarification/purification procedure ______________________________________
  - Antibiotics (if any) used during preparation _______________________________
  - Identification of preservatives/stabilizers (if any) _________________________

**Tests on monovalent pool**

*Determination of infectivity (potency)*
  - Method ________________________________
  - No. of vaccine-quality embryonated eggs or cell cultures inoculated __________
  - Date of determination ________________________________
  - Results ____________________________________________

*Determination of attenuation*
  - Method __________________________________________________________________
  - Date of determination ________________________________
  - Results ____________________________________________

*Determination of identity of virus*
  - Method __________________________________________________________________
  - Date of determination ________________________________
  - Results ____________________________________________
### Test for adventitious virus agents

- **Method**
- **Date of test**
- **Results**

### Test in vaccine-quality embryonated eggs (for egg-based vaccine)

- **Method**
- **Date of test**
- **Results**

### Test in cell cultures

- **Method**
- **Date of test**
- **Results**

### Test for adventitious bacterial agents

- **Method**
- **Date of test**
- **Results**

### Test for adventitious fungal agents

- **Method**
- **Date of test**
- **Results**

### Test for adventitious mycoplasma agents

- **Method**
- **Date of test**
- **Results**

### Test for adventitious mycobacterial agents

- **Method**
- **Date of test**
- **Results**

### Test for chemicals used in production (may be performed on final bulk)

- **Method**
- **Date of test**
- **Results**
Test for residual DNA (for cell-culture vaccine)
Method ______________________________________________________
Date of test ____________________________________________________
Results _______________________________________________________

Final bulk
Name and address of manufacturer _______________________________________
Identification of final bulk ____________________________________________
Identification of monovalent virus pool(s) used to prepare final bulk ________
Date of manufacture ________________________________________________

Control of final bulk
Test for infectivity (potency) (may be performed on final bulk)
Method ______________________________________________________
Date of test ____________________________________________________
Results _______________________________________________________

Test for sterility
Method ______________________________________________________
Date of test ____________________________________________________
Results _______________________________________________________

Test for total protein
Method ______________________________________________________
Date of test ____________________________________________________
Results _______________________________________________________

Test for ovalbumin (for egg-based vaccine)
Method ______________________________________________________
Date of test ____________________________________________________
Results _______________________________________________________

Test for residual DNA (for cell-culture vaccine)
Method ______________________________________________________
Date of test ____________________________________________________
Results _______________________________________________________

Test for chemical used in production
Method ______________________________________________________
Date of test ____________________________________________________
Results _______________________________________________________

Test for sterility
Control of final product

Test for infectivity (potency)
Method ___________________________
Date of test ___________________________
Results ___________________________

Test for identity
Method ___________________________
Date of test ___________________________
Results ___________________________

Test for sterility
Method ___________________________
Date of test ___________________________
Results ___________________________

Test for endotoxin
Method ___________________________
Date of test ___________________________
Results ___________________________

Test for residual moisture (if applicable)
Method ___________________________
Date of test ___________________________
Results ___________________________

Inspection of final containers
Results ___________________________

Other tests ___________________________

Additional comments (if any) ___________________________

A sample of a completed final container label and package insert should be attached.
Certification by producer
Name of head of production (of final product) ______________________

Certification by head of the quality assurance department taking overall responsibility for production and control of the final product:

I certify that Lot No. ________________ of Influenza Vaccine (Human, Live Attenuated) for Intranasal Administration, whose number appears on the label of the final container, meets all national requirements¹ and satisfies Part A of the Requirements for Biological Substances No. 17, revised 1990.

Signature  ________________________________
Name typed  ________________________________
Date  ________________________________

Certification by the national regulatory authority
If the vaccine is to be exported, provide a copy of the certificate from the national regulatory authority as described in section D.2, a label of a final container, and a leaflet of instructions to users.

¹ If any national requirement(s) is(are) not met, specify which one(s) and indicate why release of the lot has nevertheless been authorized.
Appendix 2

Reference laboratories

WHO Collaborating Centres for Reference and Research on Influenza

- WHO Collaborating Centre for Reference and Research on Influenza, Melbourne, Australia
- WHO Collaborating Centre for Reference and Research on Influenza, National Institute of Infectious Disease, Tokyo, Japan
- WHO Collaborating Centre for Reference and Research on Influenza, National Institute for Medical Research, London, England
- WHO Collaborating Centre for the Surveillance, Epidemiology and Control of Influenza, Centers for Disease Control and Prevention, Atlanta, GA, USA
- WHO Collaborating Centre for Studies on the Ecology of Influenza in Animals, St Jude Children's Research Hospital, Memphis, TN, USA

Essential regulatory laboratories

- Therapeutic Goods Administration, Immunology and Vaccines, Canberra, Australia
- National Institute of Infectious Disease, Tokyo, Japan
- National Institute for Biological Standards and Control, Potters Bar, England
- Center for Biologics Evaluation and Research, Division of Viral Products, Food and Drug Administration, Rockville, MD, USA

WHO reference laboratories for diagnosis of influenza A/H5 infection

- WHO Collaborating Centre for Reference and Research on Influenza, Melbourne, Australia
- Department of Microbiology, Faculty of Medicine, University of Hong Kong, China, Hong Kong Special Administrative Region
- National Influenza Centre, Centre for Health Protection, China, Hong Kong Special Administrative Region
- Virology and Zoonotic Disease Research Program, US Naval Medical Research Unit 3 (NAMRU-3), Cairo, Egypt
WHO Collaborating Centre for Reference and Research on Influenza, National Institute for Medical Research, London, England
Institute Pasteur, Unité de Génétique Moléculaire des Virus Respiratoires, Paris, France
National Institute of Virology, Pune, India
WHO Collaborating Centre for Reference and Research on Influenza, National Institute of Infectious Disease, Tokyo, Japan
Federal State Research Institution, State Research Centre for Virology and Biotechnology VECTOR, Novosibirsk Region, Russian Federation
WHO Collaborating Centre for the Surveillance, Epidemiology and Control of Influenza, Centers for Disease Control and Prevention, Atlanta, GA, USA
WHO Collaborating Centre for Studies on the Ecology of Influenza in Animals, St Jude Children's Research Hospital, Memphis, TN, USA
Appendix 3

Model certificate for the release of influenza vaccine (human, live attenuated) for intranasal administration

The following lots of influenza vaccine (human, live attenuated) for intranasal administration called [trade name and/or common name of the product] __________________ produced by [name and address of manufacturer] __________________ 2 in [list of manufacturing sites] __________________ 3 whose marketing authorization number is ____________ [insert marketing authorization number] and whose numbers appear on the labels of the final containers, meet all national requirements,4 the manufacturing recommendations in part A of the WHO Recommendations to assure the quality, safety and efficacy of influenza vaccines (human, live attenuated) for intranasal administration (revised 2009)5 and Recommendations for good manufacturing practice and quality assurance for biological products,6 and has been approved for release.

<table>
<thead>
<tr>
<th>Lot number (including sub-lot number and packing lot numbers if relevant)</th>
<th>Container type and number of doses per container</th>
<th>Storage conditions</th>
<th>Number of containers/batch size</th>
<th>Date of start of period of validity (e.g. manufacturing date) and/or expiry date</th>
</tr>
</thead>
</table>

As a minimum, this certificate is based on examination of the manufacturing protocol.

---

1 To be provided by the national regulatory authority of the country where the vaccines have been manufactured.
2 Name and address of manufacturer.
3 Country.
4 If any national requirement(s) is (are) not met, specify which one(s) and indicate why release of the lot(s) has nevertheless been authorized by the national regulatory authority.
5 To be published in WHO Technical Report Series, No. 977, 2013, Annex 4; with the exception of the provisions on shipping, which the national regulatory authority may not be in a position to control.
The number of this certificate is: ________________________________
The date of issue of this certificate is: ________________________________
Title of authorizing official (typed): ________________________________
Name of official (typed): ________________________________
Signature of official: ________________________________
Date: ________________________________

7 Or her or his representative.
Annex 5

Biological substances: International Standards and Reference Reagents

A list of International Standards and Reference Reagents for biological substances 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, 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 2009, the Expert Committee made the following changes to the previous list.

These substances are held and distributed by the International Laboratory for Biological Standards, National Institute for Biological Standards and Control, Potters Bar, Hertfordshire, EN6 3QG, England.

Additions

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Activity</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antigens and related substances</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poliovirus type 1 (Sabin), for MAPREC assay</td>
<td>2.06% 480A, 525C DNA per ampoule</td>
<td>First International Standard</td>
</tr>
<tr>
<td>Poliovirus type 1 (Sabin), restriction enzyme cleavage control for MAPREC assay</td>
<td>85.2% 480A, 525C DNA per ampoule</td>
<td>First WHO Reference Reagent</td>
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<tr>
<td>Poliovirus type 1 (Sabin), low-mutant virus reference for MAPREC assay</td>
<td>1.84% 480A, 525C DNA per ampoule</td>
<td>First WHO Reference Reagent</td>
</tr>
<tr>
<td>Poliovirus type 1 (Sabin), high-mutant virus reference for MAPREC assay</td>
<td>2.56% 480A, 525C DNA per ampoule</td>
<td>First WHO Reference Reagent</td>
</tr>
<tr>
<td>Human papillomavirus type 16, antibodies</td>
<td>5 International Units per ampoule</td>
<td>First International Standard</td>
</tr>
<tr>
<td><strong>Bordetella pertussis</strong>, monoclonal antibody against fimbrial antigen 2, for serotyping</td>
<td>No assigned value</td>
<td>First 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><em>Bordetella pertussis</em>, monoclonal antibody against fimbrial antigen 3, for serotyping</td>
<td>No assigned value</td>
<td>First International Standard</td>
</tr>
<tr>
<td>Bacilli Calmette-Guérin (BCG) vaccine, Danish sub-strain</td>
<td>CFU content of $7.29 \times 10^6$ and an ATP content of 56.06 ng per ampoule</td>
<td>First WHO Reference Reagent</td>
</tr>
<tr>
<td>Bacilli Calmette-Guérin (BCG) vaccine, Tokyo sub-strain</td>
<td>CFU content of $49.37 \times 10^6$ and an ATP content of 217.60 ng per ampoule</td>
<td>First WHO Reference Reagent</td>
</tr>
<tr>
<td>Diphtheria toxoid (adsorbed)</td>
<td>213 IU per ampoule</td>
<td>Fourth International Standard</td>
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</table>

**Blood products and related substances**

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<tr>
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<th>Activity</th>
<th>Status</th>
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<tbody>
<tr>
<td>Blood coagulation factor VIII, concentrate</td>
<td>9.4 IU/ampoule</td>
<td>Eighth International Standard</td>
</tr>
<tr>
<td>Blood coagulation factor VIII and von Willebrand factor (VWF), plasma</td>
<td>Factor VIII: C – 0.68 IU/ampoule Factor VIII: antigen – 1.04 IU/ampoule von Willebrand factor: antigen – 1.00 IU/ampoule von Willebrand factor: ristocetin cofactor – 0.87 IU/ampoule von Willebrand factor: collagen binding – 1.03 IU/ampoule</td>
<td>Sixth International Standard</td>
</tr>
<tr>
<td>Streptodornase</td>
<td>3200 IU per ampoule</td>
<td>Second International Standard</td>
</tr>
<tr>
<td>Unfractionated heparin</td>
<td>2145 IU/ampoule</td>
<td>Sixth International Standard</td>
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</table>

**Cytokines, growth factors and endocrinological substances**

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<tr>
<th>Preparation</th>
<th>Activity</th>
<th>Status</th>
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</thead>
<tbody>
<tr>
<td>Chorionic gonadotrophin, human</td>
<td>179 IU per ampoule for calibration of immunoassays; 162 IU per ampoule for calibration of bioassays; 0.39 nmol per ampoule for calibration of immunoassays in molar units</td>
<td>Fifth International Standard</td>
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<tr>
<td>Parathyroid hormone, 1-84, human, recombinant</td>
<td>100 micrograms per ampoule</td>
<td>First 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><strong>Diagnostic reagents</strong></td>
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</tr>
<tr>
<td>HIV-2 RNA</td>
<td>1000 IU/ml</td>
<td>First International Standard</td>
</tr>
<tr>
<td>Hepatitis B virus genotypes for NAT-based assays</td>
<td>No assigned values</td>
<td>First International Reference Panel</td>
</tr>
<tr>
<td>Parvovirus B19 DNA genotypes</td>
<td>No assigned values</td>
<td>First International Reference Panel</td>
</tr>
<tr>
<td>Thromboplastin, human, recombinant, plain</td>
<td>International Sensitivity Index (ISI) value of 1.082</td>
<td>Fourth International Standard</td>
</tr>
<tr>
<td>Soluble transferrin receptor, recombinant</td>
<td>21.7 mg/l and 303 nmol/l when reconstituted with 0.50 ml distilled or deionized water</td>
<td>First WHO Reference Reagent</td>
</tr>
<tr>
<td>Prader-Willi and Angelman syndromes</td>
<td>No assigned values</td>
<td>First International Genetic Reference Panel</td>
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<tr>
<td>Fusion gene BCR-ABL</td>
<td>No assigned values</td>
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