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, and endocrinological substances. The second part of the report, of particular relevance to manufacturers and national regulatory authorities, contains guidelines on quality, safety and efficacy of live attenuated rotavirus vaccines; DNA vaccines; a biosafety risk assessment for production and quality control of human influenza pandemic vaccines; recommendations for inactivated rabies vaccines produced in cell substrates and embryonated eggs; for whole cell pertussis vaccine; and for production, control and regulation of human plasma for fractionation.

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.
The World Health Organization was established in 1948 as a specialized agency of the United Nations serving as the directing and coordinating authority for international health matters and public health. One of WHO’s constitutional functions is to provide objective and reliable information and advice in the field of human health, a responsibility that it fulfills in part through its extensive programme of publications.

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

To ensure the widest possible availability of authoritative information and guidance on health matters, WHO secures the broad international distribution of its publications and encourages their translation and adaptation. By helping to promote and protect health and prevent and control disease throughout the world, WHO’s books contribute to achieving the Organization’s principal objective — the attainment by all people of the highest possible level of health.

---

**SELECTED WHO PUBLICATIONS OF RELATED INTEREST**

**WHO Expert Committee on Biological Standardization**
Fifty-fifth report.
WHO Technical Report Series, No. 932, 2006 (137 pages)
web site www.who.int/biologicals

**WHO Expert Committee on Biological Standardization**
Fifty-fourth report.
WHO Technical Report Series, No. 927, 2005 (154 pages)

**WHO Expert Committee on Biological Standardization**
Fifty-third report.
WHO Technical Report Series, No. 926, 2004 (109 pages)

**WHO Expert Committee on Biological Standardization**
Fifty-second report.
WHO Technical Report Series, No. 924, 2004 (234 pages)

---

The WHO Technical Report Series makes available the findings of various international groups of experts that provide WHO with the latest scientific and technical advice on a broad range of medical and public health subjects. Members of such expert groups serve without remuneration in their personal capacities rather than as representatives of governments or other bodies; their views do not necessarily reflect the decisions or the stated policy of WHO. An annual subscription to this series, comprising about six such reports, costs Sw. fr. 132.– or US$ 106.– (Sw. fr. 92.40 in developing countries).

For further information, please contact Marketing and Dissemination, World Health Organization, 20 avenue Appia, 1211 Geneva 27, Switzerland (tel.: +41 22 791 2476; fax: +41 22 791 4857; e-mail: bookorders@who.int).

---

Further information on these or other WHO publications can be obtained from Marketing and Dissemination, World Health Organization, 1211 Geneva 27, Switzerland
The production of rabies vaccine should be conducted by dedicated staffs who do not handle other infectious microorganisms, animals, or tissue cultures in the same working day.

Steps should also be taken to minimize the risks of transmission of rabies virus from the production facility to the outside environment.

A.3 Control of source materials

A.3.1 Substrates for virus production

Rabies vaccines may be produced in human diploid cells, in continuous cell lines, in primary hamster kidney cells or in primary chick embryo fibroblast cells. For human diploid and continuous cell lines section 3.1.1 should apply; for primary hamster kidney cells section 3.1.2 should apply; for primary chick embryo fibroblasts section 3.1.3 should apply; for embryonated duck eggs section 3.1.4 should apply. Section 3.1.5 applies to all types of cell substrates.

A.3.1.1 Diploid cells and continuous cell lines

The use of a diploid cell or continuous cell line for the manufacture of rabies vaccines should be based on the cell seed system. The cell seed should be approved by the national regulatory authority. The maximum number of passages (or population doublings) by which the working cell bank is derived from the cell seed should be approved by the national regulatory authority.

WHO has established a cell bank of Vero cells characterized in accordance with the requirements in the report of the WHO Expert Committee on Biological Standardization (15), which is available as a well characterized starting material to manufacturers for preparation of their own master and working cell seeds on request to the Coordinator, Quality Assurance and Safety of Biologicals, WHO, Geneva, Switzerland.

A.3.1.1 Identity test

Cell seed should be characterized according to the Requirements for animal cell lines used as substrates for production of biologicals (15), as appropriate to continuous cells or human diploid cells.

The testing performed on a replacement master cell bank (derived from the same clone or from an existing master or working cell bank) should be the same as for the establishment of the initial master cell bank, unless a justified exception is made. The WCB should be identified by means of, for example, biochemical (e.g. isoenzyme analysis), immunological, and cytogenetic marker tests, approved by the national regulatory authority.
A.3.1.2. Animals

Syrian hamsters 10–14 days old may be used as the source of kidneys for cell culture. Only hamster stock approved by the national regulatory authority should be used as the source of tissue and should be derived from a closed, healthy colony. A closed colony is a group of animals sharing a common environment and having their own caretakers who have no contact with other animal colonies. The animals are tested according to a defined programme to ensure freedom from specified pathogens, including the absence of antibodies to these pathogens. When new animals are introduced into the colony, they should be maintained in quarantine in vermin-proof quarters for a minimum of two months and shown to be free from these specified pathogens. The parents of animals to be used as a source of tissue should be maintained in vermin-proof quarters. Neither parent hamsters nor their progeny should previously have been used for experimental purposes, especially those involving infectious agents. The colony should be monitored for zoonotic viruses and markers for contamination at regular intervals.

At the time the colony is established, all founder animals should be tested to determine freedom from antibodies to the following pathogens: microorganisms pathogenic for hamsters (e.g. Mycobacterium tuberculosis, lymphoma virus, papilloma virus, polyomavirus, adenoviruses and retroviruses), lymphocytic choriomeningitis virus, pneumonia virus of mice, reovirus type-3, minute virus of mice, Sendai, Hantaan virus, SV-5, Toolans H-a, mouse polio, mouse hepatitis virus, and Kilham rat virus. Hamster antibody production (HAP), mouse antibody production (MAP) and rat antibody production (RAP) tests should also be performed. A test for retroviruses using a sensitive polymerase chain reaction (PCR) based reverse transcriptase (Rtase) assay also should be included. The results of such assays need to be interpreted with caution because Rtase activity is not unique to retroviruses and may derive from other sources, such as retrovirus-like elements that do not encode a complete genome (20). Nucleic acid amplification tests for retrovirus may also be used. A PCR test for hamster polyoma virus should be used on a selected number of hamster tissues, especially kidneys, to qualify the colony, and at intervals thereafter.

After the colony is established, it should be monitored by testing a representative group of animals. The choice of tests and testing procedures for monitoring as well as the appropriate number of animals should be approved by the national regulatory authority. In addition, the colony should be screened for the presence of pathogenic bacteria, including mycobacteria; fungi and mycoplasma. This should be performed in all of the animals over a defined period of time. The screening programme should be approved by the national regulatory authority.
Any animal that dies should be investigated to determine the cause of death. If the presence of an infectious agent is demonstrated in the colony, the national regulatory authority should be informed and the manufacture of vaccine should be discontinued. In this case, manufacture should not be resumed until a thorough investigation has been completed and precautions have been taken against the infectious agent being present in the product, and only then with the approval of the national regulatory authority.

At the time of kidney harvest, the animals should be examined for the presence of any abnormalities and if kidney abnormalities or other evidence of pathology is found, the affected animals are not to be used for production of rabies vaccine.

Each group of control cultures derived from a single group of animals used to produce a single virus harvest should remain identifiable as such until all testing, especially for adventitious agents, is completed.

### A.3.1.2.2 Cell cultures for virus propagation

Kidneys derived from animals which comply with the guidelines set out in section A.3.1.2.1. should be dissected and minced under conditions approved by the national regulatory authority. A primary cell suspension is obtained after trypsin digestion and this is distributed into cell culture vessels with growth medium.

Penicillin and other Beta-lactam antibiotics should not be used at any stage of manufacture. Minimal concentrations of suitable antibiotics such as kanamycin and neomycin may be used if approved by the national regulatory authority.

### A.3.1.3 Chicken eggs used for primary chick embryo fibroblast preparation

If the vaccine is to be produced in primary chick embryo fibroblasts, the eggs to be used should be from a closed, specific-pathogen-free flock. This flock should be monitored at regular intervals for agents pathogenic to birds. These include *Mycobacterium avium*, fowl pox virus, avian leukosis virus (ALV) and other avian retroviruses, Newcastle disease virus and other avian parainfluenza viruses, avian encephalomyelitis virus, infectious laryngotracheitis virus, avian reticulo-endotheliosis virus, Marek’s disease virus, infectious bursal disease virus, avian adenoviruses — group 1, avian infectious bronchitis virus, avian nephritis virus, avian orthoreoviruses, chicken anaemia virus, egg drop syndrome virus, influenza A virus, turkey rhinotracheitis virus, *Haemophilus paragallinarum*, *Salmonella enterica* Gallinarum and relevant *Mycoplasma* spp.

In some countries, all birds are bled when a colony is established, and thereafter 5% of the birds are bled each month. The resulting serum samples are screened for antibodies to the relevant pathogens. Any bird that dies should be investigated to determine the cause of death.

The flock must not have been vaccinated.
A3.1.4 **Embryonated duck eggs**

If the vaccine is to be produced in the embryonated eggs of ducks, the eggs to be used should be from a closed, specific-pathogenic-free flock. The flock should be regularly monitored for agents pathogenic to ducks. The day old ducklings should come from an establishment or a hatchery where duck virus enteritis, duck viral hepatitis, *Salmonella enteritidis*, *Salmonella anatum*, *Salmonella aertrycke*, avian tuberculosis, psittacosis-orinthosis, fowl cholera (pasteurellosis), egg drop syndrome, avian influenza (type A) adenovirus group III (EDS), avian rotavirus, avian encephalomyelitis, avian J virus, infectious serositus (new duck disease), coliform septicaemia, spirochaetosis (duck tick fever), reticuloendotheliosis virus and Newcastle disease have not been reported during the past 12 months.

In some countries, all ducks are bled when a colony is established, and thereafter 5% of the ducks are bled each month. The resulting serum samples are screened for antibodies to the relevant pathogens. Any ducks that dies should be investigated to determine the cause of death.

Live vaccine against avian influenza should never have been used in the supply flocks. If any other vaccine is used the name and nature of the vaccine, source of vaccine and date of vaccination should be reported.

A3.1.5 **Cell culture medium**

Serum used for the propagation of cells should be tested to demonstrate freedom from bacteria, fungi and mycoplasmas, according to the requirements given in Part A, sections 5.2 and 5.3 of the revised Requirements for Biological Substances No. 6 (23), and from infectious viruses. Suitable tests for detecting viruses in bovine serum are given in Appendix 1 of the Recommendations for Production and Control of Poliomyelitis Vaccine (Oral) (24).

Validated molecular tests for bovine viruses may replace the cell culture tests of bovine sera. As an additional monitor of quality, sera may be examined for freedom from phage and for an acceptable limit of bacterial endotoxin.

Irradiation may be used to inactivate potential contaminant viruses.

The acceptability of the source(s) of any components of bovine, sheep or goat origin used in culture media should be approved by the national regulatory authority. These components should comply with current WHO guidelines in relation to animal transmissible spongiform encephalopathies (16).

Human serum should not be used. If human albumin is used it should meet the revised Requirements for the collection, processing and quality control of blood, blood components and plasma derivatives (Requirements for
Biological Substances No. 27) (17), as well as current WHO guidelines in relation to human transmissible encephalopathies (16).

The use of human albumin as a component of a cell culture medium requires careful consideration due to potential problems with the validity period of albumin in relation to the intended long-term storage of rabies vaccines.

Penicillin and other beta-lactam antibiotics should not be used at any stage of manufacture. Minimal concentrations of suitable antibiotics such as kanamycin and neomycin may be used if approved by the national regulatory authority.

Nontoxic pH indicators may be added, e.g. phenol red in a concentration of 0.002%. Only substances that have been approved by the national regulatory authority may be added.

If trypsin of animal origin is used for preparing cell cultures it should be tested and found free of cultivable bacteria, fungi, mycoplasmas and infectious viruses, especially bovine or porcine parvoviruses, as appropriate. The methods used to ensure this should be approved by the national regulatory authority.

The source(s) 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 in relation to animal transmissible spongiform encephalopathies (16).

A.3.2 Virus seed

A.3.2.1 Strains of virus

The strains of rabies virus used in the production of all seed lots should be well characterized, laboratory adapted and attenuated\(^1\) with stable biological characteristics. The strains should be identified by historical records including the information on its origin. They should have been shown, to the satisfaction of the national regulatory authority, to yield safe and immunogenic vaccines when inactivated.

Vaccine strains used for production of vaccines derived from cell substrates and embryonated eggs known to induce protection in humans against rabies include, but are not restricted to, the Pitman Moore virus, Pasteur Virus, the Vnukovo –32, the Flury LEP, and the CTN. These are examples from

---

\(^1\) Previously used term “fixed” is based on the defined time in which clinical symptoms of the disease appear in animals when inoculated intracerebrally (e.g. rabbits, mice and sheep). This was obtained by serial passaging of the virus in rabbits. Although this characteristic reflects attenuation of virus in an appropriate animal model it is not a guarantee of the suitability of a rabies virus for production of vaccines for human use. The latter should be based on the immunogenicity and safety of an inactivated virus in humans.
some of currently licensed vaccines and should not be interpreted as a recommendation.

The choice of virus, its full characterization and adaptation to the production substrate should be justified in the overall evaluation of rabies vaccines for licensing.

Vaccine strains should be characterized by molecular and serological methods, including the use of monoclonal antibodies for the characterization of rabies virus. This should also include animal inoculation. In addition, sequencing of at least the glycoprotein and nucleoprotein genes of master or working seed should be considered.

All master and working seed lots should comply with the current guidelines to minimize the risks of transmission of animal transmissible spongiform encephalopathies (16).

A.3.2.1 Virus seed lot system

Vaccine production should be based on the virus seed lot system. The working virus seed lot should be not more than five passages removed from the master virus seed lot, which should have been thoroughly characterized. Vaccines should be made from the working seed lot without further intervening passage. Virus seed lots should be maintained either in the dried or in the frozen form and each lot should be stored separately. If frozen, the seed lots should be kept continuously at a temperature below –60 °C.

A.3.2.2 Tests on virus seed lots

Seed lots should have been shown, to the satisfaction of the national regulatory authority, to be capable of yielding vaccine that meets all the manufacturing requirements listed here.

The virus master and working seed lots should be identified as rabies virus by methods approved by the national regulatory authority.

Monoclonal antibodies which react specifically with rabies virus nucleocapsid and glycoprotein may be used to identify the virus as rabies.

All master and working seed lots should comply with the current guidelines to minimize the risks of transmission of animal transmissible spongiform encephalopathies (16).

A.3.2.1 Tests for bacteria, fungi and mycoplasmas

Each virus seed lot should be tested for bacterial, fungal, and mycoplasmal contamination by appropriate tests according to Part A, section 5.2 of the Requirements for biological substances no.6. General requirements for the sterility of biological substances (23).
The virus master or working seed lots used for the production of vaccine in cell substrates or embryonated eggs should be free from detectable adventitious agents. Seed lots produced in cell substrates should comply with the recommendations in Part A. Section 3.1.1 applies to seed production in human diploid or continuous cell lines; section 3.1.2 applies to primary hamster kidney cells; and section 3.1.3 applies to seed lots produced using primary chick embryo fibroblasts; section 3.1.4 applies to embryonated duck eggs.

For these tests the virus should first be neutralized by a specific anti-rabies serum.

The individual tests on the seed virus should be designed to satisfy the requirements of the national regulatory authority. The anti-rabies serum should be free of known adventitious viruses.

**Tests in suckling mice.** A sample of the virus suspension should be tested for the presence of adventitious agents pathogenic to mice by intracerebral inoculation of 0.01 ml and intraperitoneal inoculation of at least 0.1 ml into at least 10 suckling mice. The mice should be less than 24 hours old and originate from more than one litter. They should be observed daily for at least 14 days. All mice that die within the first 24 hours following inoculation or that show signs of illness should be examined for evidence of viral infection. This should be done macroscopically and by subinoculation of appropriate tissue suspensions by the intracerebral and intraperitoneal routes into at least five additional suckling mice, which should be observed daily for 14 days.

In some countries, in addition, a blind passage is made of a suspension of the pooled emulsified tissue (minus skin and viscera) of all mice surviving the original 14-day test.

The virus seed passes the test if at least 80% of the mice originally inoculated remain healthy and survive the observation period, and if none of the mice shows evidence of infection with any adventitious agent attributable to the virus seed.

**Tests in adult mice.** A sample of the virus suspension should be tested for the presence of adventitious agents pathogenic to mice by intracerebral inoculation of 0.03 ml, intraperitoneal inoculation of at least 0.25 ml, and inoculation of 0.01 ml into the footpad in at least 20 adult mice, each weighing 15–20 g. The mice should be observed for at least 4 weeks. All mice that die within the first 24 hours of inoculation or that show signs of illness should be examined for evidence of viral infection. This should be done macroscopically by direct observation and by subinoculation of appropriate tissue suspensions by the intracerebral and intraperitoneal routes into at least five additional mice, which should be observed for 3 weeks.
The virus seed passes the test if at least 80% of the inoculated animals remain healthy and survive the observation period, and if none of the mice shows evidence of infection with any adventitious agent attributable to the virus seed.

*Tests in cell cultures.* The neutralized seed virus should be tested for freedom from adventitious viruses in three sensitive cell culture systems:

— the cell line used for production;
— a different cell line from a different species; and
— human diploid cells.

Ten millilitres of the neutralized seed virus should be inoculated into each cell system and the cells incubated at 35–37 °C for 14 days.

For virus seeds produced in human diploid cells, cell cultures should be held for 28 days for the detection of cytomegalovirus.

The cells should be observed microscopically for cytopathic changes. At the end of the observation period, the cells or fluids should be tested for haemadsorbing viruses and other adventitious agents as specified in Part A, sections 4.1.1.1. and 4.1.1.2. For the tests to be valid, at least 80% of the culture vessels should be available and suitable for evaluation at the end of the observation period. For the seed virus to be satisfactory, no cytopathic changes or adventitious agents should be detected. Control cell cultures should be included in the tests.

Additional tests if chick cell cultures are used for production of virus seed

If chicken cell cultures are used, a sample of fluids pooled from the control cultures should be tested for avian retroviruses such as avian leukosis virus, by a method approved by the national regulatory authority.

A test for retroviruses using a sensitive polymerase chain reaction (PCR)-based reverse transcriptase (Rtase) assay may be used. The results of such assays need to be interpreted with caution because Rtase activity is not unique to retroviruses and may derive from other sources, such as retrovirus-like elements that do not encode a complete genome (22). Nucleic acid amplification tests for retrovirus may also be used.

Virus content

A titration of the virus content of each seed lot should be made. Such titrations may be done either in cell culture or by the inoculation of mice. If mice are used, they should be inoculated by the intracerebral route with 0.03 ml quantities of suitable dilutions of the virus seed lot. Although the previously recommended end-point for this *in vivo* titration was death of the mice, it is reasonable instead to use clinical signs of paralysis as the
end-point and to kill the animals when they reach this stage. Mice that show no signs of rabies infection such as ruffled fur, slow and shaky movements or paralysis should be observed for 14 days. The minimum titre of the seed should be specified by the manufacturer, according to the product, cells and virus strain.

A.3.2.1.2.4 Additional tests if duck embryos are used for production of virus seed

If duck embryos are used for the production of virus seed, tests for *Mycobacteria* and avian viruses should be performed.

A.4. **Control of vaccine production**

A.4.1 **Control (of) cell cultures**

Penicillin and other beta-lactams should not be used at any stage of manufacture.

Minimal concentrations of other suitable antibiotics, such as kanamycin and neomycin, may be used where approved by the national regulatory authority.

At least 5% or 500 ml, whichever is greater, of the cell suspension at the concentration employed for seeding vaccine production cultures, should be used to prepare control cultures.

If bioreactor technology is used, the national regulatory authority should determine the size and treatment of the cell sample to be examined.

A.4.1.1 **Tests of control cell cultures**

The control cell cultures should be treated in a similar way to the production cell cultures, but they should remain uninoculated so that they can be used for the detection of extraneous viruses.

The control cell cultures should be incubated under the same conditions as the inoculated cultures for two weeks or until the last harvest of virus from the production cultures—whichever is the later—and should be examined during this period for evidence of cytopathic changes. For the test to be valid, not more than 20% of the control cell cultures should have had to be discarded because of accidental contamination or damage.

At the end of the observation period, the control cell cultures should be examined for degeneration caused by infectious agents. If this examination, or any of the tests specified in this section, shows evidence of the presence in a control culture of any adventitious agent, the rabies virus grown in the corresponding inoculated cultures shall not be used for vaccine production.
A.4.1.1.1 Tests for haemadsorbing viruses

At the end of the observation periods, 25% of the control cells should be tested for the presence of haemadsorbing viruses by using guinea-pig erythrocytes. If the guinea-pig erythrocytes have been stored, the duration of storage should not have exceeded 7 days and the temperature of storage should have been in the range of 2–8 °C.

In tests for haemadsorbing viruses, calcium and magnesium ions should be absent from the buffer medium.

In some countries the national regulatory authority requires that tests for haemadsorbing viruses should also be done with erythrocytes from other species, including human beings (blood group 0), monkeys, and chickens (or other avian species).

The results of all tests should be noted after incubation of the erythrocytes with the cultured cells for 30 minutes at 0–4 °C and again after a further incubation for 30 minutes at 20–25 °C. For the test with monkey erythrocytes, the results should be noted a third time, after a final incubation for 30 minutes at 34–37 °C.

A.4.1.1.2 Tests for other adventitious agents in control cell fluids

At the end of the observation period a sample of the pooled fluids from each group of control cultures should be tested for adventitious agents. Ten millilitres of each pool should be tested in the same cells, but not the same batch of cells, as those used for virus production, and additional 10 ml samples of each pool should be tested in human cells and at least one other sensitive cell system.

The inoculated cultures should be incubated at 35–37 °C and should be observed for at least 14 days.

For the tests to be valid, at least 80% of the culture vessels should be available and suitable for evaluation at the end of the test period.

If any cytopathic changes due to adventitious agents occur in any of the cultures, the virus harvest produced from the batches of cells from which the control cells were taken should be discarded.

A.4.1.1.3 Identity test (cell line)

At the production level, and for vaccines produced in human diploid cells or continuous cell lines only, the cells should be identified by using one of the methods specified in current requirements for the use of animal cells as in vitro substrates for production of biologicals (15). The method(s) should be approved by the national regulatory authority.

Methods for identity testing include, but are not limited to, biochemical (e.g. isoenzyme analysis), immunological (e.g. HLA assays), cytogenetic
tests (e.g. for chromosomal markers), and tests for genetic markers (DNA finger-printing).

A.4.1.4 Additional tests on control cells if avian embryo cells are used for production

A sample of the control fluid taken at the end of the observation period of the control cell cultures should be tested for avian retroviruses such as avian leukemia virus, by a method approved by the national regulatory authority.

In some countries the complement fixing test (COFAL) is used for detecting avian leukemia viruses, and liver or kidney cell cultures of embryos are used for detecting adenoviruses. A test for retroviruses using a sensitive PCR-based RTase assay may be used. The results of such assays need to be interpreted with caution because RTase activity is not unique to retroviruses and may derive from other sources, such as retrovirus-like elements that do not encode a complete genome (nucleic acid amplification tests for retrovirus may also be used).

Only those cells shown to be free from contamination should be used.

A.4.1.5 Additional tests on control cells if other cell cultures are used

When other cell substrates are used for the growth of rabies virus, additional appropriate tests should be considered.

A.4.2 Control of production in embryonated duck eggs

A.4.2.1 Control of (uninoculated) embryonated duck eggs

A sample of 2% of, but in any case not less than 20 and not more than 50, uninoculated embryonated eggs from the batch used for vaccine production should be incubated under the same conditions as the inoculated eggs. At the time of virus harvest, the uninoculated eggs should be processed in the same manner as the inoculated eggs, and the extract from the control embryos should be shown to be free from haemagglutinating agents and from adenoviruses, avian retroviruses such as avian leukemia virus, and other extraneous agents by tests approved by the national regulatory authority.

A test for retroviruses using a sensitive PCR-based RTase assay may be used. The results of such assays need to be interpreted with caution because RTase activity is not unique to retroviruses and may derive from other sources, such as retrovirus-like elements that do not encode a complete genome (22). Nucleic acid amplification tests for retrovirus may also be used.

A.4.2.2 Single harvests from embryonated duck eggs

After the eggs have been incubated for a suitable period they should be inoculated with seed virus. After further incubation for a suitable period, only living, typical duck embryos should be harvested with aseptic precaution.
The time of harvest should be defined starting from the inoculation of the virus and should not be more than 14 days. Embryos inoculated at the same time and harvested together may be pooled and the viral harvest kept separate until completion of the sterility test (Part A, section 4.2.1.).

A.4.3 **Control of single virus harvests and purified bulk material**

After inoculation of the production cells with the virus working seed lot, neither inoculated nor control cell cultures should at any time be at a temperature outside the range approved by the national regulatory authority for the defined incubation periods. The optimal range for pH, multiplicity of infection, cell density and time of incubation should be established for each manufacturer, and be approved by the national regulatory authority.

The appropriate time for harvest should be defined as number of days after virus inoculation and should be approved by the national regulatory authority.

It is advisable that the inoculated cell cultures are processed in such a manner that each virus suspension harvested remains identifiable as a single harvest and is kept separate from other harvests until the results of all the tests described in part A sections 4.1 and 4.2 have been obtained.

Only virus harvests satisfying the recommendations below should be pooled and used in the preparation of the inactivated virus harvest.

A.4.3.1 **Sterility tests on single virus harvests**

A sample removed from each virus harvest should be tested for bacterial and fungal contamination by appropriate methods, according to Part A, section 5.2 of the *Requirements for biological substances no.6. General requirements for the sterility of biological substances* (23). In addition, test on mycoplasma contamination should be performed.

Any virus harvest in which contamination is detected must be discarded.

A.4.3.2 **Identity**

The single harvest contains virus that should be identified as rabies virus using specific antibodies.

A.4.3.3 **Virus titration for infectivity**

Each virus harvest or pool of harvests should be tested for infectivity in a sensitive assay. Both mice and cell culture of defined sensitivity are suitable for testing infectivity. Manufacturers should set an in-house specification for the titre of each harvest or pool of harvests.

A.4.3.4 **Determination of antigen content**

Assays for determination of glycoprotein antigen for determining the antigen content of the final bulk have been shown to be suitable for
monitoring consistency of production. Such assays include the single radial immunodiffusion and enzyme immunoassay (EIA) test. Selection of antibodies and other reagents is of critical importance.

A.4.35 Monitoring consistency of production

Virus titre, as well as determination of the antigen content mentioned above, are appropriate parameters for monitoring consistency of production. Therefore, internal specifications should be set.

A.4.36 Purification and/or concentration and of virus harvests

One or more single harvests may be purified and/or concentrated by methods demonstrated to yield safe, potent and immunogenic vaccine. The virus harvest should be inactivated by a validated method at a defined stage of the process which may be before, during or after any concentration and purification.

The process should be approved by the national regulatory authority and should be shown to give consistent results.

A.4.37 Test for residual cellular DNA

For viruses grown in continuous cell lines, purified bulk should be tested for residual cellular DNA. The purification process should be shown to consistently reduce the amount of host cell DNA. As recommended in the Requirements for the use of animal cells as in vitro substrates for the production of biologicals (15), the amount of residual cell DNA should be less than 10 ng per purified human dose. The assay for determination of residual cell DNA with defined sensitivity for detection of specified levels should be approved by the national regulatory authority. The specification set for the level of residual DNA should comply with current WHO guidelines for cell substrates.

A.4.38 Test for residual animal serum

If animal serum is used during production, the concentration of bovine serum albumin (BSA) may be used as an indicator of animal serum in the purified bulk which should not be greater than 50 ng per human dose or its equivalent.

In some countries, tests are carried out to estimate the amount of residual animal serum in the final vaccine.

A.4.39 Tests for residual materials

Each manufacturer should demonstrate, by testing each virus purified bulk or by validation of the manufacturing process, that any residual materials used in manufacture are consistently reduced to a level acceptable to the national regulatory authority.
A.4.4 **Inactivation procedure**

A.4.4.1 **Methods and agents**

The methods and agents used for inactivation should be validated and approved by the national regulatory authority. Given that presence of virus aggregates may result in ineffective inactivation, great care should be taken to avoid this. If it is not possible, virus aggregates should be removed before starting the inactivation procedure.

Chemical or physical means, such as filtration, may be used to remove aggregates.

If clarification is performed on a crude virus suspension, it is advisable to start inactivation within 24 hours.

In the case of vaccine produced in embryonated eggs the method should also be shown to inactivate avian leukosis viruses, as demonstrated by tests in tissue culture, or, in the case of vaccine produced in the primary hamster kidney cells, any adventitious agent that may be present, as demonstrated by tests using tissue culture or by animal inoculation.

The method used should be shown to be consistently effective in the hands of the manufacturer. The kinetics of inactivation should be demonstrated by the manufacturer to be consistently effective using at least five consecutive batches. The total inactivation time to complete inactivation should be determined. The inactivation time used routinely should be at least double the period required to inactivate the virus completely.

As a part of validation of the inactivation process, virus samples taken at appropriate times, should be inoculated immediately into the sensitive substrate (e.g. mice, cell cultures), to determine the inactivation curve. This provides information on the reproducibility of the inactivation process.

Various methods for inactivating rabies virus have been used with success. The concentration of the inactivating chemical, the temperature, and the length of time necessary for inactivation must be defined by each manufacturer for a particular production process. Satisfactory vaccines may be prepared by treating virus suspensions from tissue culture at 2–8 °C with beta-propiolactone at a dilution of 1:3500 to 1:5000 for 24 h, or until inactivation is complete, as demonstrated by the results of the test for effective inactivation specified below.

The conditions for storage of concentrated bulk intermediates should to be validated and approved by the national regulatory authority.

Formaldehyde may also be used as an inactivating agent in the production of rabies vaccines. Tests for free formaldehyde should be performed at appropriate intervals and the concentration maintained
at specified levels. Ultraviolet irradiation may be used to facilitate the inactivation process.

**A4.4.2 Tests for effective inactivation**

Each purified bulk material should be tested in an appropriate test system for effective inactivation of the virus before the addition of preservatives and other substances. The sensitivity of the assay should be determined according to the rabies virus used for production and the most sensitive assay should be used. Tests should be performed immediately after inactivation.

If samples are not tested immediately after inactivation they should be stored frozen at < −60°C. The conditions of storage should be validated to confirm that there is no loss of virus titre. If test is performed at a later stage of production, appropriate biosafety levels should be maintained.

The rabies virus amplification test, for testing for the presence of live virus, should be performed in the cell culture used for vaccine production or a type of cell line demonstrated to be of greater sensitivity. The national regulatory authority should approve the cell line and the method used. Manufacturers are encouraged to use cells such as Vero cells, BHK-21 and neuroblastoma cells which are known to be highly sensitive to rabies virus.

The rabies virus amplification test should be done as follows. At least 25 ml of bulk vaccine corresponding to at least 25 human doses should be inoculated on five cell cultures of the type used for vaccine production, or a type of at least equal sensitivity. At least 3 cm² of cell sheet should be used per millilitre of vaccine. After adsorption of the inoculum for an appropriate time, medium should be added such that the ratio of medium to vaccine is not more than 1:3. The cultures should be observed for at least 21 days. The cell cultures may be stained directly for the presence of rabies virus by immunofluorescence. Otherwise, 5 millilitres of each culture fluid should be pooled on days 14 and 21 and 0.03 ml of this pool should be inoculated intracerebrally into each of 20 weanling mice of 12–15 g. These mice should be observed for 14 days. Any symptoms caused by rabies virus should be confirmed by the immunofluorescence assay. At the end of the observation period, no cytopathic effects should be detected.

A test involving the use of immunofluorescence for the detection of cells infected with rabies virus at day 21, which is shown to be as sensitive as mice inoculation and approved by the national regulatory authority may be used. A specification for the proportion of cells checked by immunofluorescence should be set and approved by the national regulatory authority. The inclusion of trypsinisation of the cells on day
7 should be considered as this may increase the sensitivity of the amplification assay.

For certain products such as purified duck embryo vaccine (PDEV) the virus may not be adapted to growth in cell culture. Virus amplification test may, therefore, be performed in the same substrate as the one used for production. The virus is inoculated in yolk sac in pre-incubated eggs. Absence of virus can be confirmed by the fluorescent antibody test.

The bulk material passes the test if the product is shown, to the satisfaction of the national regulatory authority, to be free from residual live virus.

A.4.5 Preparation and control of the final bulk

A.4.5.1 Preservatives and other substances added

In preparing the final bulk, only adjuvant, preservatives and other substances such as human albumin 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 at the concentration used.

If beta-propiolactone has been used for inactivation, the procedure should be such that this chemical is not detectable in the final bulk. The test used for determination of beta — propiolactone should be of defined sensitivity, performed at the time intervals appropriate for the kinetics of inactivation for the vaccine in question. The test should be approved by the national regulatory authority.

No antibiotics should be added to rabies vaccine for human use after the virus has been harvested.

A.4.5.2 Antigen content of the final bulk

Assays for determination of glycoprotein antigen of the final bulk have been shown to be suitable for monitoring consistency of production. Such assays include the single radial immunodiffusion and EIA test. Selection of antibodies and other reagents is of critical importance.

Since the presence of adjuvant may affect results, it is advisable to perform the assay before the adjuvant is added. Alternatively, antigen may be eluted from the adjuvant prior to assay.

Some manufacturers test glycoprotein content of the purified, concentrated bulk to determine the dilution of the bulk to be used in the preparation of the final bulk.

A.4.5.3 Sterility tests

Each final bulk should be tested for sterility according to Part A, section 5.2 of the revised Requirements for biological substances no. 6. General requirements for the sterility of biological substances (23).
A.5. **Filling and containers**

The requirements concerning filling and containers given in Good Manufacturing Practices for Biological Products (20) should apply to vaccine filled in the final form.

Care should be taken to ensure that the material of which the container is made does not adversely affect the virus content of the vaccine under the recommended storage conditions.

A.6. **Control tests on final product**

Samples should be taken from each filling lot for the tests described in the following sections.

A.6.1 *Identity test*

An identity test should be performed on at least one labelled container from each filling lot by an appropriate method.

The test for potency described in Part A, section 6.5 may serve as an identity test.

A.6.2 *Sterility test*

Each filling lot should be tested for bacterial and fungal sterility according to Part A, section 5.2 of the revised *Requirements for biological substances no. 6. General requirements for the sterility of biological substances* (23).

A.6.3 *General safety (innocuity) test*

Each final lot should be tested for unexpected toxicity (sometimes called abnormal toxicity) using a general safety (innocuity) test approved by the national regulatory authority.

This test may be omitted for routine lot release once consistency of production has been well established to the satisfaction of the national regulatory authority and when good manufacturing practices are in place. Each lot, if tested, should pass a test for general safety.

A.6.4 *Antigen content*

If not done on the final bulk (4.3.2), antigen content should be determined and be within limits approved by the national regulatory authority.

A.6.5 *Potency test of vaccine in final containers*

The potency of each final lot should be determined. Before being tested, dried vaccine should be reconstituted to the form in which it is to be used in humans.
The NIH test as described in Laboratory techniques in rabies (10) should be used to evaluate consistency of production of the vaccine in question. This should also be used to test product stability for the purpose of establishing shelf life as well as to calibrate reference preparations.

In this test mice are immunized and subsequently challenged with rabies virus. The test is conducted by vaccinating groups of mice, on two occasions, 7 days apart, with dilutions of an appropriate reference material calibrated against the International Standard for Rabies Vaccine and vaccine being tested. Seven days after the last vaccination, the immunized animals and a control group of mice are challenged intracerebrally with the > 5 LD$_{50}$ of challenge virus standard (CVS). The titre of the challenge virus should be confirmed by inoculation of at least three tenfold dilutions into further groups of mice. The mice are observed for 14 days and the 50% effective dose (ED$_{50}$) of the reference and test vaccine is determined on the basis of survival rate of mice. Humane endpoints may also be used if validated.

The potency of the test vaccine in IU should be determined by comparing the ED$_{50}$ of the test vaccine with that of the reference vaccine calibrated in IU by comparison with the International Standard for Rabies Vaccine using appropriate statistical methods.

The assay with defined criteria for validity and test procedure, the method for statistical calculation together with the minimum number of assays to be performed for adequate interpretation of the results and the confidence limits of the assay should be approved by the national regulatory authority. In particular, calibration of reference vaccine against the International Standard as well as use, storage and handling of CVS should be well defined in the approved test procedure.

The confidence limits of the assay should be in the range of 25–400%.

The number of tests to be undertaken on each batch is dependent on the consistency of assays in an individual laboratory. If consistency of testing in a laboratory is well demonstrated, one assay may be sufficient. However, additional assays may increase the precision of the potency estimate.

The potency should be at least 2.5 IU per single human dose.

In some countries, more than one assay is performed. In this case, the estimated geometric mean potency is based on two valid tests and should be at least 2.5 IU per human dose.

In some countries, a modified NIH test is in use. Following licensing, and once consistency in production and quality control of the vaccine has been further confirmed on a continuous basis over at least two years, the determination of potency in routine lot release may, with the approval of the national regulatory authority, be based on the modified
NIH assay, based on a single dilution. This assay will provide qualitative (or semi-quantitative) results.

Several prerequisites and conditions should be considered before designing a single dilution assay:

- The one dilution assay is advantageous when vaccine lots consistently give a lower limit for the estimated potency well in excess of 2.5 IU per single human dose. This is more likely to be consistently achieved where the antigen content of the final container vaccine is based on the assay of rabies glycoprotein content.

- This assay is suitable for testing a large number of batches each year, in particular for testing several batches at the same time.

- Consistency of testing results is essential.

- Several factors such as virus strain, the homogeneity of the CVS challenge preparation and the strain and quality of mice may affect reproducibility of the results of tests.

The following criteria for validity of a single dilution assay should be taken into account:

- The full dilution assay should be well established with high percentage of valid results for defined period of time.

- Reference vaccine and CVS should have a good record of values within the specified range for the laboratory in question.

- Sub-potent vaccines should be included in the validation.

- Acceptance and rejection criteria must be defined.

To further confirm consistency on a continuous basis, the potency of about 10 recent batches of vaccine should be tested using the full dilution assay. If potency expressed in IU is within the specified range of values and if the expectations of linearity and parallelism are consistently satisfied, then fewer doses may be used and the assumptions of linearity and parallelism need not be tested in each assay.

A one-dilution assay is based on the same principles for evaluating the response as the three-dilution assays. The assay involves the selection of a dose of the reference vaccine, expressed as a fraction of 2.5 IU (i.e., of the minimum potency of a single human dose), that elicits a minimum protective effect in mice, and comparing its effect with the response elicited by the same fraction of a human dose of the test vaccine. If the response to the test vaccine is significantly greater than the response to the reference vaccine ($P \leq 0.05$), the potency of the test vaccine is satisfactory.
One dilution assays provide assurance that the lower limit of the estimated potency is in excess of the minimum requirement. A disadvantage of such an approach is that strictly quantitative estimates of vaccine potency will not be possible.

Lot release based on a simplified approach will require periodic review to ensure that the validity of all procedures is maintained. The timing of the review should be decided on a case by case basis depending on the number of batches of vaccine produced annually and/or performed regularly (at least every 2 years), as agreed by the national regulatory authority.

If a batch of vaccine fails to meet the specification set for the modified test, a full NIH mouse protection test should be performed.

Manufacturers are encouraged to support data generated by NIH potency assay by the determination of antigen content using an in vitro assay in order to ensure overall consistency of production.

The design of the test as well as statistical analysis of the data should be approved by the national regulatory authority.

A suitable challenge strain, CVS-11, is available upon request from the World Health Organization, Geneva, Switzerland. Such a request should be approved by the relevant national regulatory authority.

A.6.6 Ovalbumin content

For vaccines produced in embryonated eggs only, the ovalbumin content of each filling lot, if not done on the final bulk, should be determined and be within limits approved by the national regulatory authority.

A.6.7 Residual moisture test on freeze-dried vaccine

The residual moisture in a representative sample of each freeze-dried lot may be determined by a method approved by the national regulatory authority. The upper limit of moisture content should be specified by the national regulatory authority. Generally, moisture levels of less than 3% are considered satisfactory.

A.6.8 Test for pyrogenic substances

Each final lot should be tested for pyrogenic substances. The test should be approved by the national regulatory authority.

A.6.9 Test for residual animal serum protein

A sample of the final lot should be tested to verify that the level of bovine serum albumin in the final reconstituted vaccine is less than 50 ng per human dose.
A.6.10 **Adjuvant**

If an adjuvant has been added to the vaccine, its content should be determined by a method approved by the national regulatory authority.

Where aluminium compounds are used the concentration of aluminium should not be greater than 1.25 mg per single human dose. If other substances are used as adjuvant or those with adjuvanted effect, a specification should be set.

When aluminium hydroxide is used as the adjuvant, the degree of adsorption should be determined in the final bulk. This should not be less than 95%.

A.6.11 **Preservative**

If a preservative has been added to the vaccine, the content of preservative should be determined by a method approved by the national regulatory authority.

The amount of preservative in the vaccine dose should be shown neither to have any deleterious effect on the antigen nor to impair the safety of the product in humans. The preservative, its use at different stages of the manufacturing process as well as the residual amount present in the product should be approved by the national regulatory authority.

If any modification of preservative content in an already licensed vaccine is made, general principles for vaccine evaluation described in the WHO Guidelines on regulatory expectations related to the elimination, reduction or replacement of thiomersal in vaccines, should be followed (25).

A.6.12 **Inspection of final containers**

Each container in each filling lot should be inspected, and those showing abnormalities should be discarded.

A.6.13 **Test for residual cellular DNA**

For viruses grown in continuous cell lines, the final product should be tested for residual cellular DNA if this test has not been carried out at final bulk stage (section A.4.3.7).

A.7. **Records**

The recommendations given in *Good manufacturing practices for biological products* (20) should apply.

A.8. **Samples**

Vaccine samples should be retained as recommended in Good Manufacturing Practices for biological products (20, Annex 1).
A.9. **Labelling**

The recommendations given in *Good manufacturing practices for biological products* (20) should apply, with the addition of the following.

The label on the container or package should include the following information:

— the designation of the strain of rabies virus contained in the vaccine;
— the minimum potency of vaccine determined by NIH test and expressed in IU per human dose;
— the substrate used for the preparation of the vaccine;
— the method used for inactivating the virus;
— the nature and amount of stabilizer, preservative or additive present in the vaccine;
— the volume and nature of diluent;
— the use of vaccines after reconstitution if the vaccine is in the dried form;
— the expiry date should be indicated on both the primary and secondary packaging.

It is desirable that the label carry the names both of producer and of the source of the bulk material, if the producer of the final vaccine did not prepare it. The nature and residual amount of the antibiotics present in the vaccine, if any, may be included.

A.10. **Distribution and shipping**

The recommendations given in *Good manufacturing practices for biological products* (20) should apply.

A.11. **Stability, storage and expiry date**

A.11.1 **Stability**

Stability evaluation is an important part of the quality assessment. The purpose of stability studies is to ensure that the vaccine at the end of its shelf life, storage period or period of use, still has the required characteristics supporting quality, safety and efficacy.

A.11.1.1 **Stability for licensing**

Studies that support stability of a vaccine for the purpose of licensing have to be performed as real time real condition studies. Stability-indicating parameters should be carefully selected. They should always include, but should not be limited to, the potency test as part of real time studies under conditions recommended for storage. Tests should be conducted to determine the loss of potency at appropriate time intervals during storage. Final containers from at least three batches of vaccine derived from different bulks should be tested on the expiry date to demonstrate stability during storage.
Data from accelerated stability testing for a product stored for limited periods at temperatures that may affect stability could support preliminary data from ongoing real time stability studies but should not replace them. However, further data on stability to support shelf life of the product should be based on long-term stability studies under real conditions and should be submitted to the national regulatory authorities for approval. Any modification of the shelf life approved as part of licensing requires additional stability data to support the proposed modification and should be approved by the relevant national regulatory authority. Following licensure, stability should be monitored throughout the proposed shelf-life.

A.11.1.2 Stability for lot release
There is no additional value in performing an accelerated stability test for the purpose of lot release.

A.11.1.3 Stability at different stages of manufacturing process
Stability testing should be performed at different stages of production, namely on single harvests, final bulk and final lot. Stability indicating parameters should be selected according to the stage of production. It is advisable to assign a shelf-life to all materials during vaccine production, in particular intermediates such as single harvests, purified bulk and final bulk.

A.11.1.4 Stability for clinical trial approval
For vaccines under development, stability data, such as those described under 11.1.1, are expected for the purpose of clinical trial approval. However, stability data generated for a more limited period are acceptable at this stage.

Appropriate documentation to support the stability profile of a vaccine should be submitted to the competent national regulatory authority at all stages mentioned above.

A.11.2 Storage conditions
Recommended storage conditions and the defined maximum duration of storage should be based on stability studies as described above and approved by the national regulatory authority. For rabies vaccines, both liquid and freeze-dried, a temperature of 2–8 °C has been found satisfactory. This should ensure that the minimum potency specified on the label of the container or package will be maintained after release until the end of the shelf-life, if the conditions under which the vaccine is stored are in accordance with those stated on the label.

A.11.3 Expiry date
The expiry date should be defined on the basis of shelf life supported by the stability studies as described above (section A.11) and approved by the national regulatory authority.
A.12. **Intradermal route of administration**

Vaccines to be administered by the intradermal route should meet the same quality, safety and efficacy specifications as defined in the WHO recommendations for production and control for vaccines for intramuscular use. This means that the potency of such vaccines, if reconstituted in the volume intended for intramuscular use, should be at least 2.5 IU per single dose. In addition, manufacturers should provide clinical evidence that the vaccine is immunogenic and safe when administered intradermally.

In some countries a volume of 0.1ml per intradermal site has been found appropriate due to practical aspects of vaccine administration.

Ideally, vaccines intended to be administered by intradermal route should be developed for this purpose. This includes appropriate studies in which the immunogenicity and safety of vaccines are demonstrated by testing vaccine, the potency of which is assigned for an intradermal dose.

For vaccines originally developed for intramuscular administration, intradermal use should be supported by nonclinical and clinical data (see sections B and C). In addition, potency should be assigned for the intradermal dose.

Rabies vaccines formulated with an adjuvant should not be administered intradermally.

Intradermal injections must be administered by staff trained in this technique.

Further details on immunization regimens and practices to be followed when a vaccine is to be administered by the intradermal route are available in the report of the WHO Expert Consultation on Rabies (7).

**Part B. Nonclinical evaluation of new rabies vaccines**

Preclinical testing is a prerequisite for the initiation of clinical trials in humans and includes immunogenicity studies (proof of concept) and safety testing in animals. The vaccine lots used in preclinical and nonclinical studies should be adequately representative of the formulation intended for use in the clinical investigation and, ideally, preclinical testing should be done on the same lots as proposed for the clinical trials. If this is not feasible, then these lots should be comparable with respect to potency, stability, and other characteristics of quality. Details of the design, conduct, analysis and evaluation of nonclinical data are available in the *WHO Guidelines for nonclinical evaluation of vaccines* (26).

If a new rabies vaccine is intended to be used intradermally, the issue of appropriate formulation for this purpose should be addressed early in its
development. Dose–response and minimum potency for induction of a protective immune response in a relevant animal model should be demonstrated.

Part C. Clinical evaluation of rabies vaccines

C.1 Evaluation of new rabies vaccines for licensing

C.1.1 General considerations for the clinical assessment of rabies vaccines

The clinical development programme for rabies vaccines should evaluate their use for pre-exposure and post-exposure prophylaxis including different vaccination schedules and routes of administration, the onset, extent and duration of protection, and the need for and timing of booster vaccination. Clinical trials should adhere to the principles described in the Guidelines for good clinical practice (27) as well as to those formulated for design, conduct and analysis of vaccine clinical trials described in the WHO Guidelines for clinical evaluation of vaccines (28). All clinical trials should be approved by the relevant national regulatory authority.

For ethical reasons, it is impossible to conduct placebo controlled clinical efficacy studies involving an unvaccinated group that might be or has been exposed to rabies infection. Efficacy has been demonstrated previously in well designed but uncontrolled studies for tissue culture- and avian-derived vaccines in individuals exposed to rabies infection. Long-term evidence has shown that vaccines that met the minimum WHO potency requirement of 2.5 IU per dose induce adequate immunogenicity and protection. An antibody concentration of at least 0.5 IU per ml on days 14 and 28 or 30, after initial vaccination, is generally considered to be adequate. Therefore a satisfactory concentration of neutralizing antibodies could be used as a predictor of clinical efficacy. Nevertheless every effort should be made to obtain information on the protective capacity of vaccines during their actual use.

The clinical development programme must be tailored to the type of vaccine, taking into consideration the particular formulation, including any adjuvant content, the production process and intended use of a vaccine. For example, vaccines containing antigens, adjuvants or other components with which there is little or no previous experience in humans will require more extensive clinical study than those that closely resemble licensed vaccines.

C.1.2 Characterization of vaccine lots for clinical trials

By the beginning of the later stages of clinical development, a vaccine should have been fully characterized in terms of its physicochemical and biological properties and the final manufacturing process. Final release and end of shelf-life specifications and batch release testing procedures may not have been established at this stage since they may partly depend on the total clinical data.
Consistency of manufacturing for the vaccine lots used in clinical trials should be demonstrated and well documented. These lots should be adequately representative of the formulation intended for marketing. At a minimum, candidate vaccines for clinical trials should be prepared under conditions of good manufacturing practice for clinical trial material (29). Full GMP will be required at the later stages of clinical development (19, 20). All the lots to be used for clinical trials should be released by national control laboratories.

Any change in the manufacturing process during vaccine development should be assessed carefully regarding any possible impact on the safety, immunogenicity and likely efficacy of the vaccine and the need for additional nonclinical and clinical investigations (see section 1.7 below).

Similarly if the vaccine has been the subject of a transfer of production clinical data may be required to assess its safety, immunogenicity and likely protective efficacy

C.1.3 Immunogenicity studies

Dose-finding studies to identify appropriate regimens for induction of protective immune responses should be performed.

Initial immunogenicity studies should be performed in healthy adult volunteers who have not been exposed to rabies and have not been previously vaccinated. After the vaccine has been proven to be immunogenic in rabies naïve healthy adults, further studies should be conducted to demonstrate immunogenicity in target populations according to the intended use:

Pre-exposure prophylaxis—persons resident in endemic areas should be enrolled in the trial. The population should include the elderly and persons with different vaccine histories to establish the suitability of the vaccine for naïve and previously immunized persons. If the vaccine is to be licensed for pre-exposure prophylaxis of children then adequate data should be obtained in various age groups.

Post-exposure prophylaxis—these studies can only be done in high risk areas and populations and should follow production of immunogenicity data as for pre-exposure prophylaxis. Subjects known or thought likely to have been exposed to rabies infection should be vaccinated and followed up to test for immunogenicity and efficacy. For the initial evaluation of new vaccines, schedules recommended by WHO should be used (7). For post-exposure prophylaxis regimens, the following schedules for antibody testing are recommended as a minimum: days 0, 14, 28 or 30, 90, 180, 360. It is imperative to include a blood sample taken on day 0 and 7 in order to identify and exclude previously vaccinated subjects.
For new rabies vaccines intended to be administered intradermally, the suitability of the formulation for this purpose should be tested in the target population (see section 1.8).

**C.1.4 Assessment of the immune response**

When assessing pre-exposure use, immunogenicity should be determined in terms of the time to onset, antibody titres and duration of likely protection. Variability of the immune response between subjects is an important element and should be reported wherever possible.

The ability of the vaccine to induce an immunological memory and consequently an anamnestic response after booster doses should be tested. Data generated in one study using a specific priming regimen should not be extrapolated to different regimens or routes of administration.

It would be beneficial to define the highest potency, determined by the NIH test and expressed in IU per dose, that induces further increases in antibody levels (the so called “saturation point”).

The appropriate time intervals for taking the samples should be defined taking into account study objectives. Serum samples should be divided into aliquots and stored securely so that they can be made available in the event that re-evaluation is required.

Immunogenicity should be assessed using one of the two serological assays: rapid fluorescent focus inhibition test (RFFIT) or fluorescent antibody virus neutralization (FAVN). The assay used to determine levels of neutralizing antibodies should be approved by the national regulatory authority. It has been demonstrated that the degree of homology between the strain of challenge virus used in the RFFIT to measure the immune response after vaccination and the strain of seed virus used for vaccine production profoundly affects reported rabies virus neutralizing antibodies (RVNA) values (30). The use of a heterologous challenge virus strain (CVS) may result in lower levels of neutralizing antibodies than those obtained with homologous CVS in the same assay.

**C.1.5 Quality assurance of immunogenicity testing**

It is of critical importance to perform immunogenicity testing in a laboratory with well established RFFIT/FAVN testing protocols and that implements quality assurance of testing procedures. For this purpose, the following should be in place:

- written (standard operating procedure (SOP)
- temperature monitoring and control on relevant equipment
- all equipment should be calibrated and evaluated annually. This should include all pipettes, CO² incubators, water baths, refrigerators/freezers, hoods and plate washers
• cells should be monitored to determine that they are free of mycoplasma contamination
• passage of cells should be documented
• pre-batch acceptance of all reagents to verify fitness for purpose, e.g. fetal calf serum and fluorescein isothiocyanate (FITC)-labelled anti-rabies antibody.
• virus
  — virus strain and passage history should be documented
  — virus should be passaged to avoid production of defective interfering particles
  — titre established for stock virus
  — establish working dilution
• cells
  — passage history
  — free from contamination
  — maintain same growth curve when utilized
• serum
  — store refrigerated or frozen
  — clearly labelled and destroyed adequately after usage
• assay
  — validity criteria of assay:
    • all test criteria should be documented for every test
    • back titration to ensure working virus titre is within specification
    • good cell sheet is required, reasonable confluence
    • a negative serum and a reference serum calibrated in IU must be included in all assays
    • end-point dilution of reference needs to be monitored
    • analysis of serum titres should be conducted according to documented procedures
• training and proficiency
  — all technicians need to be appropriately trained and competence demonstrated
  — participation in proficiency testing schemes (PTS) is recommended.

C.1.6 Analysis and interpretation of the data

Collection, recording, analysis and interpretation of data should be conducted according to GCP guidelines. Methodological and statistical considerations described in WHO guidelines should be taken into account (28).

Data generated in clinical trials should be submitted to the national regulatory authority as described in the Summary protocol for vaccine evaluation (28). Data should be presented stating the batch number, vaccine presentation, potency of the vaccine used, nature and volume of a diluent where appropriate, and other relevant characteristics of tested vaccine. In
addition to general statements (e.g. regarding study sites, investigators, objectives, inclusion and exclusion criteria), a number of issues specific to rabies should be described including:

— nature of exposure (WHO category 1-3);
— status and confirmation of rabid animal;
— nature of wound care;
— immunization schedule;
— details of postexposure prophylaxis (PEP) treatment including time and sites of administration of rabies immune globulin, nature, volume and other details of rabies immune globulin as a product;
— care and treatment of adverse side-effects;
— other medications given;
— control group using well-established rabies vaccine.

Immunogenicity data should include the total number and percentage of subjects in whom titres are above and less than 0.5 IU/ml, GMT with confidence interval, range of antibody titers. Safety data should be presented as total number, percentage and type of adverse events. It would be beneficial for the whole scientific community to publish clinical trial data in a peer-reviewed journal.

C.1.7 **Studies to support change in manufacturing processes**

Changes in production methods or scale-up before or following licensing will necessitate further product characterization to demonstrate comparability with the lots used in earlier studies of safety and immunogenicity. Changes that do not require clinical data should be defined in the national regulations and the national regulatory authority consulted regarding all changes prior to their implementation. However, some changes could affect the safety and immunogenicity of the vaccine and therefore it is likely efficacy and such changes should be supported by additional clinical data. The extent of the clinical data needed depends on the nature and extent of the changes made. The design of such studies rests on the demonstration of non-inferiority in terms of eliciting protective immune responses and safety (28).

C.1.8 **Studies to support a new route of administration**

Clinical data cannot be extrapolated between routes of administration and clinical trials of administration by the proposed new route should be performed. Dose finding studies should be conducted to determine the optimal intradermal dose and volume of administrations. Immunization schedules recommended by WHO are described elsewhere (7).

For vaccines already licensed for the intramuscular route of administration, the following issues should be carefully considered before proposing such a formulation for intradermal use:
• *Presence of preservative in a vaccine*: consideration should be given to the type of preservative and the residual amount in the final vaccine.

• *Potential impact of the use of opened multi-dose vials in the field*: the level of compliance with good immunization practice in a particular area/country and the actual risk of contamination should be taken into account by the national regulatory authority when this route of administration is submitted for approval.

Every effort should be made to reduce the potential risk of contamination of the vaccine in the multi-dose vials (31).

The principles of the study design are as for section 1.7 above.

**C.2 Clinical evaluation as part of postmarketing surveillance**

**C.2.1 Monitoring vaccine efficacy, effectiveness and safety**

Every effort should be made to improve scientific understanding of the protection and safety of rabies vaccines in humans by conducting active postmarketing surveillance.

It is particularly important that data are collected on any vaccine failures including detailed data on the post-exposure prophylaxis administered. Details on the proper procedure for investigation of the treatment failures are provided elsewhere (7).

Given that limited safety data are obtained in pre-licensure studies, it is very important that safety should also be monitored as part of postmarketing surveillance.

Data generated in post-marketing surveillance should be submitted to the national regulatory authority.

**Part D. Recommendations for national regulatory authorities**

**D.1 General**

The general recommendations for national regulatory authorities provided in the *Guidelines for national authorities on quality assurance for biological products* should be followed (32). These specify that no new biological substance should be licensed until consistency of production has been established.

The detailed production and control procedures as well as any change in them that may affect the quality, safety or efficacy of rabies vaccine should be discussed with and approved by the national regulatory authority.

The national regulatory authority should obtain the International Standard for potency testing and, where necessary, establish national working reference
preparation(s) calibrated against the International Standard. In addition, challenge virus standard should be obtained from a reliable source, stored and used as appropriate. The national regulatory authority should be able to provide the standard for potency testing as well as challenge virus standard on request (Part A, section 6.5).

D.2 Release and certification

A vaccine lot should be released only if it fulfils Part A of these Recommendations. Before any vaccine lot is released from a manufacturing establishment, the recommendations for consistency of production provided in Guidelines for national authorities on quality assurance for biological products (32) should be met.

A statement signed by the appropriate official of the national control laboratory should be provided if requested by a manufacturing establishment and should certify whether or not the lot of vaccine in question meets all national requirements, as well as Part A of these recommendations. The certificate should also state the lot number, the number under which the lot was released, and the number appearing on the labels of the containers. In addition, the date of the last satisfactory potency test as well as the expiry date assigned on the basis of shelf life should be stated. A copy of the official national release document should be attached.

The purpose of the certificate is to facilitate the exchange of rabies vaccine between countries. A model of a suitable certificate is given in the appendix.

Authors

The scientific basis for the revision of the Requirements published in 1981, 1987 and 1994 was developed at two meetings of the working group held at the World Health Organization, Geneva, in May 2003 and in May 2004 attended by the following people:

Dr H. Bourhy, Rabies Laboratory, National Reference Centre for Rabies, WHO Collaborating Centre for Reference and Research on Rabies, Pasteur Institute, Paris, France; Dr L. Bruckner, Institute of Virology and Immunophrophylaxis, Mittelhäusern, Switzerland; Dr W. Correa de Moura, INCQS/FIOCRUZ, Rio de Janeiro, Brazil; Dr M. Ferguson, National Institute for Biological Standards and Control, Potters Bar, England; Dr V. Grachev, Deputy Director, Institute of Poliomyelitis and Viral Encephalitis, Moscow, Russian Federation; Dr R. Gibert, Laboratories and Controls Department, French Health Products Safety Agency, Lyon, France; Dr A. Kumar, Center for Biologics Evaluation and Research, Food and Drug Administration, Rockville, MD, USA; Dr R. Levis, Center for Biologics Evaluation and Research, Food and Drug Administration, Rockville, MD, USA; Dr L. Markoff, Center for Biologics Evaluation and Research, Food and Drug Administration, Rockville,
The first draft of these revised Recommendations was prepared by Dr Ivana Knezevic, Quality and Safety of Biologicals, WHO and Dr Morag Ferguson, National Institute for Biological Standards and Control, England, following discussions at a meeting of the working group held in May 2004.

The second draft of these Recommendations was prepared by Dr Ivana Knezevic, Quality and Safety of Biologicals, WHO, taking into account information on the current manufacturing and regulatory practice obtained from a survey undertaken in 2005, outcomes of the Expert Consultation on Rabies held in 2004 as well as comments from the experts consulted.

The third draft was prepared by Dr Ivana Knezevic, Quality Assurance and Safety of Biologicals, WHO, Dr Morag Ferguson, National Institute for Biological Standards and Control, and Dr David Wood, Quality Assurance and Safety of Biologicals, WHO after an informal WHO Consultation held in July 2005, with the following participants:

Dr H. Bourhy, Rabies Laboratory, National Reference Centre for Rabies, WHO Collaborative Centre for Reference and Research on Rabies, Institut Pasteur, Paris, France; Dr D. Briggs, Department of Diagnostic, Medicine/Pathobiology College of Veterinary Medicine, Kansas State University, Manhattan, USA; Dr Cardoso de Melo, Head of Hemotherapeutic and Biological Products Unit, Ministry of Health, Brasilia, Brazil; Dr H. Chader, National Control Laboratory of Pharmaceutical Products, Ministry of Health and Population, Clinic Ahmed AROUA, Yahia, Hydra, Algeria; Dr F. Cliquet, WHO Collaborating Centre for Research and Management on Zoonoses Control, Research Laboratory on Rabies and Wild Animal Diseases, Malzeville, France; Dr G. Dong, National Institute for the Control of Pharmaceutical and Biological Products, People’s Republic of China; Dr M. Ferguson, National Institute of Biological Standards and Control, Potters Bar, Herts., England; Dr R. Gibert, Laboratoires and Controls Department, French Health Products Safety Agency, Lyon, France; Dr V. Grachev, Institute of Poliomyelitis and Viral Encephalitides, Academy of Medical Sciences of the Russian Federation, Moscow, Russian Federation; Dr E. Griffiths, Biologics and Genetic Therapies, Ottawa, Ontario, Canada; Dr R.L. Ichhpujani, Ministry of Health and Family Welfare, New Delhi, India; Dr T. Jivapaisarnpong, Division of Biological Products, Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand; Dr R. Levis, Division of Viral Products, Food and Drug Administration, Center for Biologics...
Evaluation and Research, Rockville Pike, Rockville, MD, USA; Mme L. Mahjoubi, National Control Laboratory of Medicine, Ministry of Public Health, Tunis, Tunisia; Dr H. Meyer, Paul Ehrlich Institute, Langen, Germany; Mrs D.P. Mora, National Regulatory Authority of Cuba, State Control Center for the Quality of Drugs, CECMED, Havana, Cuba; Dr Hoang Quang Huy, National Center for Quality Control of Biological Products, Hanoi, Viet Nam; Dr N. Miranda, Laguna, Manila, Philippines; Dr H. Rahman, Directorate of Drug Administration, Ministry of Health and Family Welfare, Government of the People's Republic of Bangladesh, Dhaka, Bangladesh; Dr Lucky S. Slamet, Deputy for Therapeutic Products, Narcotic, Psychotropic and Addictive Substance Control, National Agency of Drug and Food Control of the Republic of Indonesia, Jakarta Pusat, Indonesia; Dr L. Teferi, Head, Drug Administration and Control Authority, Human and Veterinary Drug Evaluation and Registration Division, Drug Administration and Control Authority, Addis Ababa, Ethiopia; Dr H. Wilde, Queen Saovabha Memorial Institute, Thai Red Cross Society and Department of Medicine, Chulalongkor University, Bangkok, Thailand; Dr E.I.P. Arisetianingsih, National Laboratory of Drug and Food Control, NADFC, Jakarta Pusat, Indonesia; Dr S. Moregeaux, Laboratories and Controls Department, French Health Products Safety Agency, Lyon, France; Dr D. Smith, Biologics and Genetic Therapies, Ottawa, Ontario, Canada; Dr M. Frazatti-Gallina, Instituto Butantan/ Fundação Butantan, São Paulo, Brazil; Dr S. Jadhav, Serum Institute of India Ltd, Pune, India; Mrs Catherine Chamberlin, European Directorate for the Quality of Medicines, Strasbourg, France; Dr C. Malerczyk, Clinical Research and Medical Affairs, Chiron Vaccines, Marburg, Germany; Dr G. Reiner, Bulk Manufacturing Germany, Chiron Vaccines, Marburg, Germany; Dr A. Sabouraud, Quality Control of Development Products, Sanofi Pasteur, Marcy l'Etoile, France; Dr J. Sokhey, WHO Regional Office for South-East Asia, World Health House, New Delhi India; Dr D. Wood, Coordinator, Quality Assurance and Safety of Biologicals, WHO, Geneva Switzerland; Dr M.P. Kieny, Director, Initiative for Vaccine Research, WHO, Geneva, Switzerland; Dr F. Meslin, Coordinator for Strategy Development and Monitoring of Zoonoses, Foodborne Diseases and Kinetoplastidae (ZFK), CPE/CDS, WHO, Geneva, Switzerland; Dr N. Dellepiane, Access to Technologies, WHO, Geneva, Switzerland; Dr A. Costa, Access to Technologies, WHO, Geneva, Switzerland; Dr C. Hernandez Rodriguez, Access to Technologies, WHO, Geneva, Switzerland; Dr I. Knezevic, Quality Assurance and Safety of Biologicals, WHO, Geneva Switzerland.

The first draft of the section on clinical evaluation of vaccines was prepared by Dr D. Briggs, Kansas State University, Manhattan, USA; Dr I. Knezevic, Quality Assurance and Safety of Biologicals, WHO, Geneva Switzerland and Dr M. Ferguson, National Institute for Biological Standards and Control, Potters Bar, England, following a discussion on 13 July 2005 attended by following people: Dr H. Wilde, Dr D. Briggs, Dr V. Gratchev, Dr M. Ferguson, Dr C. Malerczyk, Dr T. Jivapaiaarnpong, Dr R.L. Ichhpujani, Dr R. Levis, Dr J. Sokhey, Dr J. Lang and Dr I. Knezevic. Taking into account comments and suggestions made on the first draft by all participants of the consultation, in particular by the participants of the discussion held on 13 July 2005, the draft was finalized.
Acknowledgements

Acknowledgements are due to the following experts for their comments and advice on these recommendations: Dr V. Grachev, Deputy Director, Institute of Poliomyelitis and Viral Encephalitis, Moscow, Russian Federation; Dr A. Tahlan, Central Drugs Laboratory, Central Research Institute, Kasauli, India; Dr H. Bourhy, Rabies Laboratory, National Reference Centre for Rabies, WHO Collaborative Centre for Reference and Research on Rabies, Institut Pasteur, Paris, France; Dr Powell, Medicines and Healthcare Regulatory Agency, London, UK; Dr D. Briggs, Department of Diagnostic, Medicine/Pathobiology College of Veterinary Medicine, Kansas State University, Manhattan, USA; Dr S. Jadhav, Serum Institute of India Ltd, Pune, India; Mrs Catherine Chamberlin, European Directorate for the Quality of Medicines, Strasbourg, France; Dr H. Wilde, Queen Saovabha Memorial Institute, Thai Red Cross Society and Department of Medicine, Chulalongkor University, Bangkok, Thailand; Dr A. Sabouraud, Quality Control of Development Products, Sanofi Pasteur, Marcy l’Etoile, France; Dr G. Reiner, Bulk Manufacturing Germany, Chiron Vaccines, Marburg, Germany; Dr N. Miranda, Laguna, Manila, Philippines; Dr Jivapaisanpong, Division of Biological Products, Ministry of Public Health, Northabmi, Thailand; Dr F. Meslin, Coordinator for Strategy Development and Monitoring of Zoonoses, Foodborne Diseases and Kinetoplastidae (ZFK), CPE/CDS, WHO, Geneva, Switzerland; Dr N. Dellepiane, Access to Technology, WHO, Geneva, Switzerland.

References


Appendix

Summary protocol for production and testing of inactivated rabies vaccine for human use produced in cell substrates and embryonated eggs

Identification of final lot

Name and address of manufacturer ______________________________
Lot number of vaccine ________________________________
Date of manufacture of final lot ________________________________
Date of start of the potency test ________________________________
Expiry date ________________________________
Total volume of final lot ________________________________
Temperature of storage ________________________________

3. Control of source materials

3.1 Substrate for virus production

Name and identification of cell substrate ______________________________

Cell seed and WCB

Origin and short history of master and working cell bank
(date of approval by national regulatory authority) __________________
Authority that approved cell seed ______________________________
Date the MWCB was established and approved by national regulatory authority
Quantity of cell stored ________________________________
The passage level of the WCB ________________________________
Storage conditions ________________________________
Percentage of all WCB ampoules tested ________________________________

Identity test (WCB)

Method used ________________________________
Results ________________________________

Serum used in cell culture medium

Origin of serum used ________________________________
Tests performed on serum ________________________________
Results ________________________________

Trypsin used for preparation of cell cultures

Origin of trypsin used ________________________________

1 Section numbers correspond to those used in the main text of the annex.
3.2 Virus seed

Strain of virus

Name and short description of history, origin, process of attenuation, and adaptation

Date of preparation of master virus seed lot

Number of passages between isolation and primary seed

Date of preparation of working virus seed lot

Number of passages between master and working seed

Virus seed lot system

Number of subcultures between master virus seed lot and production

Method for identification of the virus seed lot

Results

Tests for bacteria, fungi, and mycoplasmas

Method used

Results

Tests for adventitious agents

Tests in suckling mice

No. of animals tested

Quantity injected

Observation period

Results (survival numbers, etc.)

Tests in adult mice

No. of animals tested

Quantity injected

Observation period

Results (survival numbers, etc.)

Tests in guinea-pigs

No. of animals tested

Quantity injected

Observation period

Results (survival numbers, etc.)

Tests in cell cultures

Methods

Results

Virus content

Method of titration

Results
4. **Control of vaccine production**

4.1 **Control of cell cultures**

*Tests for haemadsorbing viruses*

Method ____________________________________________________

Results ____________________________________________________

*Tests for other adventitious agents*

Method ____________________________________________________

Results ____________________________________________________

*Identity test (cell line)*

Method ____________________________________________________

Results ____________________________________________________

4.2 **Control of production in embryonated duck eggs**

*Control of (uninoculated) embryonated duck eggs*

Method ____________________________________________________

Results ____________________________________________________

4.3 **Control of single virus harvests and purified bulk material**

*Sterility tests of single virus harvests*

Have all the harvests included been tested for sterility? __________

Results of these tests ________________________________________

*Pooling of single virus harvests*

No. of viral harvests included __________________________________

Date of pooling _____________________________________________

*Purification of virus harvests*

Method ____________________________________________________

Degree of purity achieved _____________________________________

*Animal serum in purified bulk*

Method ____________________________________________________

Results (concentration) _______________________________________

4.4 **Inactivation procedure**

Method ____________________________________________________

Date ______________________________________________________

Temperature ________________________________________________

Tests for effective inactivation

  Volume and concentration of bulk material injected ____________
No. of mice injected ________________________________
Weight of mice ________________________________
Duration of observation ________________________________
Other animals (if used) ________________________________
Results of tests ________________________________
Rabies virus amplification test
Amount of vaccine tested (ml) ________________________________
Results ________________________________

4.5 **Preparation and control of final bulk**

*Preservatives and other substances added*
Concentration of phenol (if used) ________________________________
Other preservatives (type and concentration) ________________________________
Other substances added ________________________________

*Antigen content of the final bulk*
Method ________________________________
Results ________________________________

*Sterility tests*
Date of test ________________________________
Result ________________________________

*Other tests (chemical, biochemical)*
Type of test ________________________________
Results ________________________________

5. **Filling and containers**

Date of filling ________________________________
Quantity of containers ________________________________
Volume of vaccine per container ________________________________

*Control for defective vials*
Methods ________________________________
Results ________________________________

6. **Control tests on final product**

6.1 **Identity test**
Method ________________________________
Result ________________________________

6.2 **Sterility test**
No. of containers examined ________________________________
6.3 **Innocuity tests**

<table>
<thead>
<tr>
<th></th>
<th>Mice</th>
<th>Guinea-pigs</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of animals</td>
<td>_______________________</td>
<td>_______________________</td>
</tr>
<tr>
<td>Route of injection</td>
<td>_______________________</td>
<td>_______________________</td>
</tr>
<tr>
<td>Volume of injection</td>
<td>_______________________</td>
<td>_______________________</td>
</tr>
<tr>
<td>Date of injection</td>
<td>_______________________</td>
<td>_______________________</td>
</tr>
<tr>
<td>Date of end of test</td>
<td>_______________________</td>
<td>_______________________</td>
</tr>
<tr>
<td>Result</td>
<td>_______________________</td>
<td>_______________________</td>
</tr>
</tbody>
</table>

6.4 **Antigen content**

Type of test ____________________________

Result ____________________________

6.5 **Potency test of vaccine in final containers**

Type of test ____________________________

Date of immunization of mice ____________________________

Reference vaccine (potency) ____________________________

Challenge strain ____________________________

Date of challenge ____________________________

$ED_{50}$ test vaccine$^1$ ____________________________

$ED_{50}$ reference vaccine$^1$ ____________________________

Calculated IU/single human dose ____________________________

Confidence limits ____________________________

Results of other potency tests ____________________________

6.6 **Stability test**

Duration and temperature of incubation ____________________________

Result ____________________________

6.7 **Residual moisture test on freeze-dried vaccine**

Method ____________________________

Result ____________________________

$^1$ $ED_{50}$ quantity of vaccine that protects 50% of animals against infection with the challenge strain.
6.8 Inspection of final containers
Result ______________________________________________________

6.9 Test for pyrogenic substances
Method _______________________________________________________
Results ______________________________________________________

6.10 Test for adjuvant
Date of test ___________________________________________________
Nature and concentration of adjuvant per single human dose __________
Degree of adsorption __________________________________________

Internal certification
Certification by person taking overall responsibility for production of the vaccine
I certify that lot no. __________________ of rabies vaccine satisfies Part A of
the WHO Recommendations for inactivated rabies vaccine for human
use produced in cell substrates and embryonated eggs.
Signature _______________________________________________________
Name (typed) __________________________________________________
Date ___________________________________________________________

The protocol must be accompanied by a sample of the label and a copy of
the leaflet.

Release certification by the national regulatory authority
Whenever rabies vaccines produced in continuous cell lines are to be
exported, they should be accompanied by a release certificate from the
national regulatory authority.

Sample release certificate
I hereby certify that batch no.___________ of rabies vaccine produced by
(name of producer) in continuous cell lines meets all national requirements
as well as Part A of the WHO Recommendations for inactivated rabies
vaccine produced in cell substrates and embryonated eggs for human use.

The date of the last satisfactory potency test carried out by the national
regulatory authority is ________________________________

The final lot has been released by us under no. _________________

The number appearing on the label of the containers is _____________
Signature _______________________________________________________
Name (typed) __________________________________________________
Date ___________________________________________________________
Annex 3

Guidelines to assure the quality, safety and efficacy of live attenuated rotavirus vaccines (oral)

Introduction

General considerations

Special considerations

Use of primary cells for the production of rotavirus vaccine

Part A. Guidelines on manufacturing
  A.1 Definitions
  A.2 General manufacturing recommendations
  A.3 Control of source materials
  A.4 Control of vaccine production
  A.5 Filling and containers
  A.6 Control tests on final product
  A.7 Records
  A.8 Samples
  A.9 Labelling
  A.10 Distribution and shipping
  A.11 Storage and expiry date

Part B. Nonclinical evaluation of live attenuated rotavirus vaccines

Part C. Clinical evaluation of live attenuated rotavirus vaccines

Part D. Guidelines for national regulatory authorities
  D.1 General
  D.2 Release and certification

Authors

Acknowledgements

References

Appendix 1
Summary protocol of manufacturing and control of live attenuated rotavirus vaccines (oral)

Appendix 2
Genealogy of vaccine production process

Appendix 3
Model certificate for the release of live attenuated rotavirus vaccines

© World Health Organization
This document provides guidance to national regulatory authorities and vaccine manufacturers on the production, quality control and evaluation of safety and efficacy of live attenuated rotavirus vaccines (oral) by outlining, in detail, international regulatory expectations for product characterization. It should be read in conjunction with the WHO guidelines on nonclinical evaluation of vaccines (1), and the WHO guidelines on clinical evaluation of vaccines: regulatory expectations (2), in order to understand the whole process of vaccine evaluation. Advice that is specific to the nonclinical or clinical evaluation of live attenuated rotavirus vaccines is provided to supplement these two generic documents.

The following text is written in the form of guidelines instead of recommendations. Guidelines allow greater flexibility than recommendations with respect to expected future developments in the field.

**Introduction**

The high incidence of rotavirus disease around the world has prompted international agencies including the World Health Organization (WHO), the Global Alliance for Vaccines and Immunization (GAVI), Children’s Vaccine Program and the Rotavirus Vaccine Programme at the Program for Appropriate Technology in Health (PATH) to identify rotavirus vaccine development as one of their highest priorities. In response to this interest in the development of candidate rotavirus vaccines, WHO held an informal consultation on the Quality, Safety and Efficacy Specifications for Live Attenuated Rotavirus Vaccines in Mexico City, from 8–9 February 2005 (3). The experts present considered it timely for WHO to develop production and quality control guidelines and technical specifications for these vaccines. Draft guidelines on production and quality control specifications for live attenuated rotavirus vaccine (oral) were developed by a small drafting group established by WHO and were comprehensively reviewed by a WHO Informal Consultation on the proposed WHO Guidelines to Assure the Quality, Safety and Efficacy of Live Attenuated Rotavirus Vaccines held in Geneva, from 29–30 August 2005. After taking into account the comments made during the consultation, the revised document was submitted to and approved by the WHO Expert Committee on Biological Standardization at its 56th meeting.

The scope of this document covers live attenuated rotavirus vaccines (oral), and vaccine candidates, that are intended for international use. The aim of vaccination against rotavirus infection is to induce immunity against relevant rotavirus serotypes in one series of inoculations for the prevention of rotavirus gastroenteritis. The prevalence of multiple and different rotavirus serotypes throughout the world complicates vaccine development strategies. According to the data available in 2005, the majority of rotavirus disease
in humans is caused by five distinct serotypes of rotavirus, designated G1, G2, G3, G4 and G9 on the basis of the molecular composition of one outer coat viral protein (VP7). In the last decade, intense surveillance of rotavirus and improved laboratory assay methods have resulted in the detection of additional G serotypes that play a role in human disease. Although serotypes G1–G4 still predominate worldwide, serotypes G5, G8 and G9 have been isolated in Latin America, Africa and India respectively, and are beginning to cause a higher percentage of cases of the disease. The relative benefits of monovalent, multivalent or regional specific vaccines will remain unclear until efficacy data demonstrating heterotypic protection against relevant rotavirus serotypes become available.

The scope of this document is restricted to live vaccines, but the properties of the possible candidates are varied, particularly with respect to their degree of attenuation. This affects the extent to which they grow in culture and in the human gut and applies to strains currently being investigated as well as those studied previously. There will therefore be major quality issues specific to a particular vaccine, such as the assay of vaccine potency, the stability of the virus in production, the yield of virus and the extent of contamination by cell-derived materials. Clinical issues which will vary between candidates include the dose required to obtain immunity in recipients, the possibility of transmission to contacts of vaccinees and the genetic stability of the virus on replication in the gut of recipients. Although many of the points of possible concern considered in this document are generally applicable to rotavirus vaccines, it must be remembered that each candidate must be examined individually and that this may raise significant product-specific issues.

Several oral rotavirus vaccines are currently approved or in various stages of vaccine development. Each vaccine is the result of a unique approach in developing an attenuated phenotype and to the prevention of rotavirus disease. As more vaccines become readily available around the world, data will be provided to help in making decisions on the compositions of future vaccines. Examples of vaccines approved or in development include multivalent human–bovine and human–rhesus reassortant vaccines and monovalent vaccines containing a single attenuated human rotavirus strain; natural human–bovine reassortants or animal rotavirus strains. Vaccines for which controlled efficacy studies have been completed have demonstrated high levels of efficacy (> 80%) against severe rotavirus gastroenteritis and have also shown a range of heterotypic protection against multiple serotypes. The impact of introduction of the vaccine on the epidemiology of natural rotavirus infection is as yet unknown.

All of the vaccine candidates are claimed to be attenuated; however, the basis for the attenuation phenotype of the current vaccine candidates is
unknown. Typically the method of attenuation is to exploit the host range restriction properties of animal rotaviruses (Jennerian) by laboratory or natural (neonatal rotavirus strains) genetic reassortment with human rotavirus with the desired serotypes or insertion of multigenic mutations by serial passage in cell culture. Because laboratory markers of attenuation are not well defined and animal models demonstrating attenuation are not readily available, the claim of attenuation is based on clinical experience in human subjects. How the product will actually behave in clinical use is therefore based on clinical trials and consistency of production rather than on any specific laboratory tests. Potential laboratory markers of product consistency include information on the genetic sequence of the virus seed to show that a new seed material is similar to the previous seed and that each can be distinguished from the parent virus. Multiple passages of virus seed materials under defined cell culture conditions as well as examination of vaccine virus isolated from stool samples of vaccinees may be helpful to generate data on the conditions favouring genetic stability of the virus. Studies on consistency of production would need to take into account the variability inherent in replication of RNA virus and assess the presence of minority populations, as revealed for example by the occurrence of mixed base signals in sequencing studies. If minority populations are detected, it will be necessary to assess their biological importance, for example, by careful comparison of the level of heterogeneity between the master or working seed and higher passage levels e.g. clinical trial material.

The development of any new rotavirus vaccines must take into account the events which led to the withdrawal of one vaccine (RotaShield) from the marketplace. RotaShield was introduced in the United States in August 1998 and was withdrawn less than 1 year later when an epidemiological relationship was established between RotaShield vaccination and intussusception (IS). Early estimates suggested a risk of one case per 2500 children immunized. Re-analysis of the case–control study that examined intussusception and RotaShield revealed that the majority of the cases of IS associated with the first dose occurred in children 4 months of age or older. This did not comply with the manufacturer’s recommendation that the first dose should be given at 2 months of age and thus changed the early estimates of attributable risk of IS in the target population (4). The detailed pathogenic mechanisms for IS are unclear, but are very likely to be complex.

**General considerations**

Considerable experience in the manufacturing, testing and clinical evaluation of rotavirus vaccines has been gained from over 20 years of their development. Candidate vaccines have been and are being studied in a number of countries with diverse economic conditions and geographical
boundaries. The regulatory approvals of two rotavirus vaccines and the submission for approval of another provide a solid foundation for the preparation of these guidelines and for the continuing development of new rotavirus vaccines; they also support the global introduction of rotavirus vaccines. These experiences have enabled standards to be set for vaccine efficacy, identified safety concerns, highlighted areas of scientific weakness, and led to the introduction of new manufacturing technologies and testing strategies. Issues relevant to the development of rotavirus vaccines are discussed below.

Rotavirus is an acid-labile virus which is rapidly inactivated at an acid pH and has a half-life of less than 12 minutes at pH 2.0. Because rotavirus vaccines are intended to be administered to infants by the oral route, the virus would be inactivated by stomach gastric acid prior to reaching the site of infection in the upper gastrointestinal tract. To prevent inactivation of the virus by gastric acid, antacids or buffers are usually administered before or in combination with the oral rotavirus vaccination. The composition of the antacid and the mode of administration (in combination with vaccine or administered separately) will depend upon the biological characteristics of the vaccine virus.

The processing of live rotavirus vaccines, like that of many other live viral vaccines, involves cell disruption and, if any, incomplete purification of the virus. In-process steps for the inactivation of adventitious agents are not included for live viral vaccines, as these steps may compromise the live nature of the vaccine itself. As a result, validation of clearance of any adventitious agents may not be possible. For these reasons a comprehensive set of tests for adventitious agents and qualification of the vaccine source materials is essential as part of the vaccine safety control. As with any viral vaccines, production of rotavirus vaccines also involve cells, virus seeds and biological reagents (such as growth supplements, serum, trypsin and any virus stabilizers used in the final product). Hence, each of these vaccine source components must be shown to be free from adventitious agents. The full passage history of the seed materials used for vaccine development should identify all substrates through which they have been passed to aid the development of appropriate programmes of testing for adventitious agents. Viral seed lots should be assessed for absence of adventitious agents from all species that they may have been exposed to from isolation, through passage, and during production, including those that may be present in the raw materials used at each of these stages. The testing required will depend on available documentation on cell substrate donor, virus seed passage and derivation history and the seed virus may require purification (e.g. molecular cloning and plaque purification), if passage and derivation history is uncertain, or if it is contaminated with a known agent.
A risk assessment for transmissible spongiform encephalopathies would need to be included for the seed materials. The revised WHO Guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products (5) provide guidance on risk assessments for master and working seeds and should be consulted.

Many candidate vaccines are being developed in Vero cells. WHO has set a limit of 10 ng of DNA per human dose for parenterally administered vaccines based upon a WHO risk assessment (6) that established a $2 \times 10^8$ safety factor. For orally delivered vaccines, the acceptable limit of residual cellular DNA has not been previously established. However, recent studies in rats, which compared the uptake efficiency of Vero cell DNA following administration by the oral and intramuscular routes indicated that Vero cell DNA given orally could be found in rat tissues at a concentration of 10 000-fold less than when equivalent amounts of Vero DNA were given parenterally (7). Similarly, experiments in mice using polyoma DNA also showed a difference in uptake between the oral and parenteral routes (8). These preliminary data and the lack of evidence that cellular DNA is tumorigenic suggest that a DNA level of 100 µg/dose in an orally administered vaccine is equivalent to 10 ng in parenterally administered vaccines. In addition to the quantity of DNA, the size distribution of DNA should be evaluated during assessments of manufacturing consistency and process validation. It may be desirable for manufacturers to include steps in the process to reduce the level of high-molecular-weight cellular DNA. The national regulatory authority may require the further purification of virus harvests derived from continuous cell lines to remove cellular DNA, and/or the use of DNase treatment to reduce the size of DNA fragments. If the virus harvests are derived from diploid cell cultures, further purification is not required.

The concentration of virus can be determined by infectivity titrations, e.g. plaque-forming or focus-forming assays. However, confirming the quantity of each rotavirus serotype in the final mixture of a multivalent, as opposed to a single component, vaccine is challenging. Conventional infectivity titrations require an antibody specific to each of the vaccine components. Molecular methods of measuring virus concentration that do not require the use of specific antibody, such as quantitative real-time polymerase chain reaction (PCR), have been developed and are being evaluated. One method uses Vero cell monolayers which are seeded in 96-well plates and inoculated with dilutions of a multivalent reference standard and samples. Viral replication is allowed to proceed for 23–24 hours and the cells are then lysed by addition of Triton and one freeze–thaw cycle. Cell lysates are assayed by real-time quantitative reverse transcriptase-PCR (QPCR) and rotavirus nucleic acid is quantified (9). Individual QPCR reactions are performed employing primer/probe sets specific to each of the reassortant
strains. Virus concentration is determined by parallel line analysis between the reference standard and each sample. International titration standards do not yet exist and WHO is considering developing such reagents and their subsequent characterization by international collaborative study. It should be borne in mind however that the differences in the properties of the candidate vaccines in terms of their attenuation may mean that such reference material will be product-specific. Manufacturers should set aside a preparation for use as an in-house standard.

The real-time stability of each serotype in the final vaccine formulation should be measured to verify that the infectivity titre is at or above that level of virus required for vaccine efficacy at the end of the product shelf-life. The thermal stability of each individual serotype should be determined in an appropriate stability study programme. It is advisable to assign a shelf-life to the intermediates that are intended to be stored on the basis of stability studies. WHO is developing further guidance on this issue. Based on the results of the stability testing programme, an accelerated stability test should be done on each new batch of vaccine.

If the final product is mixed with vaccine diluent prior to use, the stability of this combination should also be determined.

The consistency of a manufacturing process for live virus vaccine can best be determined by monitoring the content and quality of virus produced throughout the process, as well as the level of impurities following processing and the stability of the final product. Manufacturers should select relevant parameters based upon the specific production process used and develop methods to establish a programme to assure the consistency of production process. For example, the amount of virus produced by a standardized production process and the amount lost during processing should be consistent; the quality of the virus produced can be assessed by its thermal stability profile; the stability of the genomic sequence through multiple cell culture passages may be evaluated; process impurities and residuals such as residual host cell protein, residual cellular DNA, endotoxin, bovine serum, trypsin and antibiotics can be measured and their reduction during processing can be monitored. If the consistency of production has been validated, the monitoring programme could be conducted based on a reduced frequency of testing, but not on a batch-to-batch basis, with the agreement of the national regulatory authority.

Special considerations

Use of primary cells for the production of rotavirus vaccine

The majority of live attenuated rotavirus vaccines that have been developed or are currently under development are produced in continuous cell lines
or diploid cells which are based on the cell bank system, so the main focus of these guidelines is on vaccines produced under such conditions. But the vaccine may also be produced in primary cells, in which case, the manufacturers should consult additional relevant guidelines or documents to ensure the safety of the final product. However, as suitable alternative cell substrates become available, primary cell cultures are less likely to be used in the future for many reasons (6). The following text provides brief guidance on issues that need to be considered if primary cells are used for rotavirus vaccine production, but there are some specific issues relating to the production of rotavirus vaccines in primary cell substrates which are not covered in these guidelines.

If vaccine is produced in primary cells obtained directly from trypsinized tissue of normal healthy animals, such animals should be of a species approved by the national regulatory authority and not have been previously employed for experimental purposes. However, primary cell cultures prepared from wild animals show a high frequency of viral contamination. The number of viruses isolated and the frequency of isolation depend on many factors, including methods of isolation, test cell systems used, number of passages, duration of incubation and co-cultivation, and are directly proportional to the duration of the incubation period of the cultures. The frequency of cell culture contamination can be reduced by careful screening of the source animals to be used in production for the absence of antibodies to the relevant viruses. The use of animals bred in a carefully controlled colony, especially those which are specific-pathogen free, is strongly recommended. In addition, the use of secondary or tertiary cells on which testing for adventitious agents could be performed will reduce the frequency of contamination of the production cell culture. Production of uninfected control cultures and the extensive testing required for relevant adventitious agents becomes more challenging when producing live rotavirus vaccines in primary cells.

A live attenuated rotavirus vaccine prepared from primary calf kidney cells has been approved for use in one country. There are no existing WHO guidelines for the production of vaccines on primary calf kidney cells. The WHO Requirements for the use of animal cells as in vitro substrates for the production of biologicals (6) provide general recommendations. The WHO Recommendations for the production and control of poliomyelitis vaccine (Oral) (10), Guidelines for the production and control of Japanese encephalitis vaccine (live) for human use (11) and Guidelines for the production of candidate live attenuated dengue virus vaccines (12) provide guidance on the use of primary cell substrates of monkey, hamster and dog kidney, respectively, and illustrate the principles that should be followed for vaccines produced in primary cell substrates from other species.
Part A. Guidelines on manufacturing

A.1 Definitions

A.1.1 International name and proper name

The international name should be Live attenuated rotavirus vaccine (LARV) (oral) with additions to indicate the virus serotype(s) of the vaccine. The proper name should be equivalent to the international name in the language of the country of origin.

The use of the international name should be limited to vaccines that satisfy the specifications formulated below.

A.1.2 Descriptive definition

Live attenuated rotavirus vaccine (oral) is a sterile vaccine preparation containing one or more virus serotypes, which have been grown through a seed lot system, prepared in a suitable approved cell substrate, formulated in a form suitable for oral administration and satisfying all the recommendations formulated in this document.

At least five types of live attenuated rotavirus vaccine have been developed and/or are under development:

— a candidate rhesus rotavirus strain live attenuated vaccine;
— a candidate human-rhesus rotavirus strain live attenuated vaccine;
— a human rotavirus strain live attenuated vaccine;
— a candidate human–bovine reassortant rotavirus strain live attenuated vaccine;
— a lamb rotavirus strain live attenuated vaccine.

The preparation should satisfy all of the specifications formulated in these guidelines.

Live attenuated rotavirus vaccine with an appropriate stabilizer may be freeze-dried or liquid.

A.1.3 International reference preparations

No international reference preparations are available at the time of preparing this document.

A.1.4 Terminology

The definitions given below apply to this document only.

Candidate vaccine. A vaccine under development which is used in human clinical trials to assess its safety and efficacy.

Virus master seed lot. A quantity of virus of uniform composition derived from an original isolate, processed at one time and passaged for a documented number of times.
Virus working seed lot. A quantity of virus of uniform composition derived from the master seed lot by a limited number of passages by a method approved by the national regulatory authority and fully characterized. The virus working seed lot is used for production of vaccine.

Cell seed. A quantity of well-characterized cells, derived from a single tissue or cell of human, animal or other origin, stored frozen in liquid nitrogen in aliquots of uniform composition, one or more of which may be used for the production of a master cell bank.

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.

Master cell bank (MCB). A quantity of fully characterized cells of human, animal or other origin stored frozen in liquid nitrogen 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.

Working cell bank (WCB). A quantity of cells of uniform composition derived from one or more ampoules of the master cell bank at a finite passage level, dispensed in aliquots into individual containers appropriately stored, usually stored frozen in liquid nitrogen, one or more of which would be used for production of cell culture. All containers are treated identically and once removed from storage, are not returned to the stock.

Production cell culture. A cell culture derived from one or more ampoules of the WCB or primary tissue used for the production of vaccines.

Adventitious agents. Contaminating microorganisms of the virus seed or cell substrate or materials used in its culture, which may include bacteria, fungi, mycoplasmas, and endogenous and exogenous viruses.

Single harvest. A virus suspension of one virus type harvested from cell cultures prepared from a single production run.

Monovalent virus pool. A homogenous pool of a number of single harvests of the same virus serotype, collected into a single vessel before clarification.

Final bulk. The finished biological preparation present in the container from which the final containers are filled. The final bulk may be prepared from one or more clarified monovalent virus pools and may contain one or more virus serotypes.
Filling lot (final lot). A collection of sealed final containers of vaccine that are homogeneous with respect to the risk of contamination during the filling process or freeze-drying. A filling lot must therefore have been filled or prepared in one working session.

Focus forming unit (FFU). Viral infectivity identified on cell monolayers using rotavirus-specific antiserum.

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

Cell culture infective dose 50% (CCID\(_{50}\)). The quantity of a virus suspension that will infect 50% of cell cultures.

Unit of infectivity (UI). Relative viral infectivity of a sample inoculated in 96-well Vero cell monolayers measured by QPCR against a defined reference standard preparation.

A.2 General manufacturing recommendations

The general manufacturing requirements contained in *Good manufacturing practices for biological products* (13) should apply to establishments manufacturing oral rotavirus vaccine, with the addition of the following:

Production steps and quality control operations involving manipulations of live rotavirus should be conducted under Biosafety Level 2 (14).

Separate areas or a campaigned programme for the manufacturing of different virus serotypes are required.

In production areas used for bulk formulation and filling, multiple serotypes may be present at the same time and these production areas may be campaigned with other vaccines provided sufficient cleaning validation and product changeover data is provided.

A.3 Control of source materials

A.3.1 Cell cultures for virus production

A3.1.1 Master cell bank and working cell bank

The use of a continuous cell line such as Vero cells (low passage, non-tumorigenic phenotype) or diploid cells such as fetal rhesus lung cells, FrHL-2 (well-defined non-tumorigenic phenotype) for the manufacture of rotavirus vaccines should be based on the cell bank system. The cell substrates and cell banks should conform with the WHO Requirements for use of animal cells as in vitro substrates for the production of biologicals (6), as appropriate to continuous cells and diploid cells, and should be approved by the national regulatory authority. The maximum number of passages (or population doublings) allowable between the cell seed and the WCB and the
production cells should be approved by the national regulatory authority. Additionally, for Vero cells, the MCB or WCB cells should be propagated to or beyond the maximum production level and be examined for the presence of retroviruses and tumorigenicity in an animal test system (6).

WHO has established a cell bank of Vero cells characterized in accordance with the requirements in the forty-seventh report of the WHO Expert Committee on Biological Standardization (6), which is available as a well-characterized source material (15) to manufacturers for preparation of their own MCB and WCB on application to the Coordinator, Quality Assurance and Safety of Biologicals, WHO, Geneva, Switzerland.

The master cell bank, which is made in sufficient quantities and stored in a secure environment is used as the source material to make manufacturer’s working cell banks. In normal practice a master cell bank is expanded by serial subculture up to a passage number (or population doubling, as appropriate) selected by the manufacturer and approved by the national regulatory authority, at which point the cells are combined to give a single pool distributed into ampoules and preserved cryogenically to form the WCB.

The manufacturer’s WCB is used for the preparation of production cell culture, and thus for production of vaccine batches.

A.3.1.1 Cell culture medium

Serum used for the propagation of cells should be tested to demonstrate freedom from bacteria, fungi and mycoplasmas, according to the requirements given in Part A, sections 5.2 and 5.3 of the General requirements for the sterility of biological substances (16), as amended in 1995 and from infectious viruses. Suitable tests for detecting viruses in bovine serum are given in Appendix 1 of the WHO Recommendations for production and control of poliomyelitis vaccine (Oral) (10).

Validated molecular tests for bovine viruses may replace the cell culture tests of bovine sera.

As an additional indicator of quality, sera may be examined for freedom from phage and endotoxin. Irradiation may be used to inactivate potential contaminant viruses.

The acceptability of the source(s) of any components of bovine, porcine, ovine or cervine origin used should be approved by the national regulatory authority. These components should comply with current WHO guidelines in relation to animal transmissible spongiform encephalopathies (5).

If trypsin is used for preparing cell cultures and aiding in virus infection, it should be tested and found free of cultivable bacteria, fungi, mycoplasmas and infectious viruses, especially bovine or porcine paroviruses, as appropriate.
The methods used to ensure this should be approved by the national regulatory authority. The trypsin should be gamma irradiated if possible.

Human serum should not be used. If human albumin is used, it should meet the revised *Requirements for the collection, processing and quality control of blood, blood components and plasma derivatives* (Requirements for Biological Substances No. 27) (17), as well as current guidelines in relation to human transmissible encephalopathies (5).

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

Nontoxic 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.

A.3.1.2 Tests on master and working cell banks

Tests on the MCB and WCB are performed in accordance with WHO *Requirements for use of animal cells as in vitro substrates for the production of biologicals* (6, 15). It is important to show that the cell banks are free of adventitious agents relevant to the species used in its derivation. Cell banks should be assessed for absence of adventitious agents that may have been present during production, including those that may be present in the source materials used at each of these stages.

Full characterization may be performed on either the MCB or on the WCB (6, 15).

A.3.2 Virus seeds

A3.2.1 Virus strains

Virus strains of rotavirus used for master and working seed lots to produce vaccines have in some cases been derived by genetic reassortment of animal rotavirus with human rotavirus with the desired serotypes or in other cases by multiple passages of human rotavirus in cell culture. The seed lot viruses should comply with the specifications of this section. Viruses may be passed in continuous, diploid, and/or primary cell lines. The candidate vaccine strains should be approved by the national regulatory authority.

- The strains of rotavirus used in the production of candidate rotavirus vaccines should be identified by historical records, which will include information on the origin of each strain, method of attenuation, whether the strains have been biologically cloned prior to generation of the master seed lots, genetic sequence information and the passage level at which attenuation for humans was demonstrated by clinical trials.
• The vaccine production strain(s) should have been shown by appropriate laboratory tests (see A.3.2.3) and/or clinical studies to yield vaccines that are safe and efficacious in humans. Only strains approved by the national regulatory authority should be used.

• The immunogenicity of the virus strains, based upon the quantity of infectious virus of each serotype present in the vaccine that induces seroconversion when susceptible individuals are immunized with the vaccine, should be established in a dose–response study. Any potential interference or potentiation between the serotypes in an infectivity assay should be evaluated prior to establishing this value. The immunizing dose established in this way serves as a basis for establishing parameters for potency at the time of release, stability and expiry date.

A.3.2.2 Virus seed lot system

The production of vaccine should be based on the virus master seed lot and virus working seed lot system. Seed lots should be prepared in the same type of cells and by the same production process as those used for production of final vaccine. Virus seed lots should be stored in a dedicated temperature-monitored refrigerator at a temperature that ensures stability and the storage arrangement should ensure appropriate security of the virus seed lots.

Seed lots of rotavirus used in the production of live attenuated rotavirus vaccine (oral) should be identified by historical records, which should include information on their origin. Only virus seed lots that are approved by the national regulatory authority should be used (see General considerations). The virus master seed lot is generally produced from a biological clone of the attenuated parent virus (animal/human reassortant or cell-culture passaged human virus) and is made in sufficient quantities and stored in a secure environment and is used as the source material to make the manufacturer’s virus working seed lots. Either the virus master seed lots or the virus working seed lots should be fully characterized and be tested extensively for adventitious agents, and approved by the national regulatory authority.

The virus master seed lot also serves as a benchmark against which to compare virus produced by subsequent passage in cell culture or shed virus following vaccination.

The manufacturer’s virus working seed lot is used for the production of vaccine batches and is prepared from the master virus seed lot. It is recommended that a large lot of virus working seed be set aside as the basic material that the manufacturer should use for the preparation of each batch of vaccine. The virus working seed lot should be prepared by defined number of passages from the virus master seed lot by a method and a passage level from the original virus seed that is established through clinical and
vaccine development studies. Once the passage level of the working seed lot is established, it may not be changed without approval from the national regulatory authority.

A.32.3 Tests on virus master and working seed lots

A.32.3.1 Identity

Each seed lot should be identified by virus type by an immunological assay or by sequencing. A molecular identity test, electropherotyping, is one recognized method for identifying rotaviruses. Comparing human and animal rotaviruses and differentiating between subgroups can be achieved through electropherotyping. Electropherotyping can be used as a means for identifying gross alterations in genomic differences between the parent wild type viruses and/or animal donor virus (if employed) and the vaccine strains. The identity of the vaccine virus is confirmed by comparing the electrophoretic profile of the vaccine virus to the RNA electrophoretic profile of a known human rotavirus serotype. Other molecular identity tests may include RNA/RNA hybridization and enzyme restriction maps or genetic sequences of PCR-amplified VP7 gene segments. The tests should be approved by the national regulatory authority.

A.32.3.2 Genotype/phenotype characterization

Genotype and phenotype stability of the seed lots upon passage should be measured using relevant assays to ensure uniformity of vaccine lots. Genetic characterization of the viruses has played a major role in the development of the vaccines already licensed or currently nearing licensure. It helps to assess the genetic and phenotypic stability of the seed lots on passage, and molecular methods have been used intensively. Other approaches including in vitro phenotypic markers might be considered.

Genetic characterization of the seed lot viruses has included determination of the nucleotide sequence of the complete viral genome, analysis of the molecular basis of the attenuated phenotype and determination of the genetic stability of the virus seed lots by comparison of the nucleotide sequence of the viral genome at different passage levels. It should be noted that full-length sequencing may not identify minority populations of variants that may be present in vaccines. In some studies the entire genomic sequence of all 11 gene segments of the virus master seed lot has been sequenced and compared with the sequence of the virus working seed lot, vaccine production lots and vaccine virus shed in stools of vaccinees. If the attenuation loci are known, sequencing around these sites can be performed instead of on the entire genome. Given the frequency of errors of the RNA replicating polymerase, base changes are likely to be found. The potential impact of any changes observed (silent or in protein-coding regions) should be evaluated.
Specific assays for markers of attenuation are still in development and the manufacturer should make an effort to investigate appropriate assays that are relevant to their vaccine.

A323.3 **Tests for bacteria, fungi and mycoplasmas**

Each virus seed lot should be tested for bacterial, fungal and mycoplasmal contamination by appropriate tests as specified in Part A, sections 5.2 and 5.3, of the *General requirements for the sterility of biological substances*, as amended in 1995 (16).

A323.4 **Tests for adventitious viruses**

Each virus seed lot should be tested in cell cultures for adventitious viruses appropriate to the origin and the passage history of the seed virus (18). Neutralization of rotavirus is necessary for many tests because the virus is cytopathogenic. Antisera used for this purpose should be shown to be free from antibodies that may neutralize specific adventitious viruses being tested for. If neutralization of rotavirus is not possible, the test sample may be passaged in trypsin-free media prior to initiating the assay, to reduce the ability of rotavirus to infect the indicator cell substrates. The cells inoculated should be observed microscopically for cytopathic changes. At the end of the observation period, the cells should be tested for haemadsorbing viruses.

Each master or working seed lot should also be tested in animals that may include guinea-pigs, mice and embryonated chicken eggs, as appropriate. For test details refer to the WHO *Requirements for measles vaccines* (Live) (19), and the *European Pharmacopoeia*, 2002 (18).

Additional testing for specific adventitious viruses may be performed, for example, using PCR amplification techniques.

A323.5 **Virus concentration**

Each seed lot should be assayed for infectivity in a sensitive assay in a cell culture system. An immunofocus or plaque forming assay may be used in MA-104, Vero or other sensitive cells to determine virus concentration. The assay is based on the visualization of infected areas (plaques or focus of infection) of a cell monolayer directly or by probing with rotavirus-specific antibodies. Results should be recorded as focus-forming units (FFU/ml) or plaque-forming units (PFU/ml).

A cell culture infectious dose assay may also be used to determine virus concentration. Results should be recorded as CCID$_{50}$/ml.

Alternatively, quantitative PCR detection of virus replication in a cell culture system may be used to provide an appropriate measure of infectivity. Results should be recorded as units of infectivity (UI/ml).
The detailed procedures for carrying out the tests and for interpreting the results should be those approved by the national regulatory authority.

A.4 **Control of vaccine production**

A.4.1 **Control of cell cultures**

From the cell suspension used to prepare cell cultures for growing attenuated rotavirus, an amount of processed cell suspension equivalent to at least 5% or 500 ml of cell suspension, whichever is greater, should be used to prepare control cultures of uninfected cells. If fermenter technology is used, the size and treatment of the cell sample to be examined should be approved by the national regulatory authority.

These control cultures should be observed microscopically for changes attributable to the presence of adventitious agents for at least 14 days after the day of inoculation of the production cultures or at the time of final virus harvest if this is later. The control cultures should be incubated under essentially similar conditions to those used for the production cultures with agreement of the national regulatory authority. At the end of the observation period, fluids collected from the control culture from each single virus harvest should be tested for the presence of adventitious agents as described below (A.4.1.2). Samples that are not tested immediately should be stored at –60 ºC or below.

If any test shows evidence of the presence of adventitious agents in control cultures, the harvest of virus from these cultures should not be used for vaccine production.

For the test to be valid, not more than 20% of the control culture vessels should have been discarded for nonspecific accidental reasons by the end of the test period.

A.4.1.1 **Tests for haemadsorbing viruses**

At the end of the observation period, cells comprising no less than 25% of the control cells should be tested for the presence of haemadsorbing viruses, 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 temperature of storage should have been in the range of 2–8 ºC.

In some countries, the national regulatory authority requires that additional tests for haemadsorbing viruses be performed using other species of red blood cells including those from humans (blood group O), monkeys and chickens (or other avian species). In all tests readings should be taken after incubation for 30 minutes at 0–4 ºC, and again after a further incubation for 30 minutes at 20–25 ºC. A further reading for the test with monkey red blood cells should be taken after an additional incubation for 30 minutes at 34–37 ºC.
For the tests to be valid, not more than 20% of the culture vessels should have been discarded for nonspecific accidental reasons by the end of the test period.

A.4.1.2  
Tests for other adventitious agents
At the end of the observation period, a sample of the pooled fluid from each group of control cultures should be tested for adventitious agents. For this purpose, 10 ml of each pool should be tested in the same cells, but not the same batch of cells, as those used for the production of virus, and additional 10-ml samples of each pool should be tested in human cells and at least one other sensitive cell system.

Each sample should be inoculated into bottles of these cell cultures in such a way that the dilution of the pooled fluid in the nutrient medium does not exceed 1 in 4. The area of the cells should be at least 3 cm² per ml of pooled fluid. At least one bottle of each kind of cell cultures should remain uninoculated to serve as a control.

The inoculated cultures and control cultures should be incubated at a temperature of 35–37 °C and should be observed for cytopathic effects for a period of at least 14 days.

For the tests to be valid, not more than 20% of the culture vessels should have been discarded for nonspecific accidental reasons by the end of the test period.

Some national regulatory authorities require that, at the end of this observation period, a subculture is made by inoculating the fluid onto fresh cells in the same culture system and observed for at least 7 days. Furthermore, some national regulatory authorities require that these cells should be tested for the presence of haemadsorbing viruses.

A.4.1.3  
Identity test
At the production level, the cells should be identified by means of tests approved by the national regulatory authority. Suitable methods are, but are not limited to, biochemical tests (e.g. isoenzyme analyses), immunological tests (e.g. HLA assays), cytogenetic tests (e.g. for chromosomal markers), and tests for genetic markers (e.g. DNA fingerprinting).

A.4.2  
Cell cultures for vaccine production
A.4.2.1  
Tests for adventitious agents
On the day of inoculation with the virus working seed lot, each cell culture and/or cell culture control should be examined for degeneration caused by infectious agents. If such examination shows evidence of the presence in a cell culture of any adventitious agents, the culture should not be used for vaccine production. Prior to infection, samples of each cell culture are removed for sterility and mycoplasma testing.
If animal serum is used for cell cultures before the inoculation of virus, it should be removed and replaced with serum-free maintenance medium, after the cells have been washed with serum-free medium, if appropriate.

A.4.2 Tests for bacteria, fungi and mycoplasmas

A volume of at least 20 ml of the pooled supernatant fluids from the production cell culture should be tested for bacterial, fungal and mycoplasmal sterility by appropriate tests as specified in Part A, sections 5.2 and 5.3, of the General requirements for the sterility of biological substances, as amended in 1995 (16).

A.4.3 Control of single harvests and monovalent virus pools

A.4.3.1 Virus inoculation

Cell cultures are inoculated with rotavirus working seed at a defined multiplicity of infection. After viral adsorption, cell cultures are fed with maintenance medium and incubated within a defined temperature range and for a defined period of time, usually established based upon the degree of cytopathic effect.

The range of multiplicity of infection, temperature, pH and time period of incubation will depend on the vaccine strain and production, a defined range should be established by the manufacturer and be approved in the marketing authorization by the national regulatory authority.

A.4.3.2 Monovalent virus pools

A virus single harvest is harvested within a defined time period post-inoculation. A monovalent virus pool may be the result of one or more single harvests (from multiple tissue culture flasks, cell factories or bioreactors) in which all harvests were derived from one or a small number of ampoules of the WCB and the same virus working seed lot recovered at the same time. If multiple single harvests are taken, each single harvest should be sampled for testing, stabilized and stored under suitable conditions until pooling. No antibiotics should be added at the time of harvesting or at any later stage of manufacture. Samples of monovalent virus pools should be taken for testing and stored at a temperature of –60 °C or below.

A.4.3.3 Tests on single harvest or monovalent virus pools

Tests may be done on single harvest or on virus pool. If the tests are done on the virus pool, the protocol should be approved by the national regulatory authority.

A.4.3.3.1 Sampling

Samples required for the testing of virus harvests should be taken immediately on harvesting prior to further processing. If the tests for adventitious agents
as described in Part A, section A.4.3.3.4, are not performed immediately, the samples taken for these tests should be kept at a temperature of –60 ºC or below and subjected to no more than one freeze–thaw cycle.

A.4.3.3.2 **Identity**

Each single harvest or virus pool should be identified as the appropriate rotavirus serotype by immunological assay or by a molecular-based assay, e.g. electropherotyping, RNA/RNA hybridization or PCR. The tests should be approved by the national regulatory authority.

A.4.3.3.3 **Tests for bacteria, fungi and mycoplasmas**

Each single harvest or virus pool should be shown to be free from bacterial, fungal and mycoplasmal contamination by appropriate tests as specified in Part A, sections 5.2 and 5.3, of the *General requirements for the sterility of biological substances*, as amended in 1995 (16).

A.4.3.3.4 **Tests for adventitious agents**

For the purposes of the recommendations set out in this section of Part A, the volume of each single harvest or virus pool taken for neutralization and testing should be at least 10 ml and should be such that a total of at least 50 ml or the equivalent of 500 doses of final vaccine, whichever is the greater, has been withheld from the corresponding final bulk.

Each virus pool should be tested in cell cultures for adventitious viruses appropriate to the passage history of the seed virus (18). Neutralization of rotavirus is necessary for many tests because the virus is cytopathogenic. Antisera used for this purpose should be shown to be free from antibodies that may neutralize the adventitious viruses being tested for. If neutralization of rotavirus is not possible, the test sample may be passaged in trypsin-free media prior to initiating the assay to reduce the ability of rotavirus to infect the indicator cell substrates. The cells inoculated should be observed microscopically for cytopathic changes. At the end of the observation period, the cells should be tested for haemadsorbing viruses.

Additional testing for specific adventitious viruses may be performed, for example using PCR amplification techniques.

A.4.3.3.5 **Virus concentration**

Each virus pool should be assayed for infectivity using a sensitive assay in cell cultures to monitor the consistency of production.

An immunofocus or plaque assay may be used in MA-104, Vero or other sensitive cells to determine virus concentration. The assay is based on the visualization of infected areas of a cell monolayer directly or by probing
with rotavirus serotype-specific monoclonal antibodies. Results should be recorded as FFU/ml or PFU/ml.

Alternatively, quantitative PCR detection of virus replication in a cell culture system may be used to provide an appropriate measure of infectivity. Results should be recorded as units of infectivity (UI/ml).

A cell culture infectious dose assay may also be used to determine virus concentration. Results should be recorded as CCID\textsubscript{50}/ml.

The detailed procedures for carrying out the tests and for interpreting the results should be those approved by the national regulatory authority.

**A.4.3.6 Tests for consistency of virus characteristics**

Tests for consistency of virus characteristics are performed during vaccine development, and process validation and not intended for batch release. Examples of studies that might be considered to characterize rotavirus are given here. Tests should be sought to compare the rotavirus in the harvest pool with the master seed virus, or suitable comparator, to ensure that the vaccine virus has not undergone critical changes during its multiplication in the production culture system. Phenotypic or genotypic characteristics (genomic sequence analysis) may be suitable. Examples of evidence to support the consistent quality of the virus produced may include in vitro growth characteristics, thermal stability profile, the ratio of infectious (triple-shelled) to non-infectious (double-shelled) particles produced and the stability of the genomic sequence through multiple cell culture passages.

Other aspects of process consistency may also be monitored and validated, such as process impurities and residuals as residual host cell protein, residual cellular DNA, endotoxin, bovine serum, trypsin and antibiotics. Their reduction during processing can be monitored to assess consistency of the manufacturing process. The reduction level should be accepted by the national regulatory authority.

**A.4.3.7 Storage**

Virus pools should be stored at a temperature that will ensure stability until formulation.

**A.4.3.4 Control of clarified monovalent virus pool (bulk)**

The monovalent virus pool may be clarified or filtered to remove cell debris and stored at a temperature that ensures stability before being used to prepare the final bulk.

**A.4.3.4.1 Sampling**

Samples of the clarified virus pool should be taken immediately after clarification and prior to further processing to ensure that no cells or cell
debris is left. Samples should also be tested as described in this section. If not tested immediately, the samples should be kept at a temperature below –50 °C until testing is done.

A.4.3.4.2 Sterility

The clarified virus pool should be tested for bacterial and fungal sterility according to the requirements in Part A, sections 5.2 of the General requirements for the sterility of biological substances (16), by acceptable methods approved by the national regulatory authority.

A.4.3.4.3 Virus concentration

Each clarified virus pool should be assayed for infectivity in a sensitive assay in cell cultures to monitor the consistency of production.

An immunofocus or plaque assay may be used in MA-104, Vero or other sensitive cells to determine virus concentration. The assay is based on the visualization of infected areas of a cell monolayer directly or by probing with rotavirus serotype-specific monoclonal antibodies. Results should be recorded as FFU/ml or PFU/ml.

Alternatively, quantitative PCR detection of virus replication in a cell culture system may be used to provide an appropriate measure of infectivity. Results should be recorded as units of infectivity (UI/ml).

A cell culture infectious dose assay may also be used to determine virus concentration. Results should be recorded as CCID$_{50}$/ml.

The detailed procedures for carrying out the tests and for interpreting the results should be those approved by the national regulatory authority.

A.4.3.4.4 Tests for residual cellular DNA

For viruses grown in continuous cells, the virus pool should be tested for residual cellular DNA. The removal process, at production scale, should be shown to consistently reduce the level of cellular DNA. The limit should be established and approved by the national regulatory authority. Based on relevant studies in animals, WHO established the acceptable limit of not more than 100 µg of cellular DNA per human dose, which is likely to provide an adequate margin of safety for orally-administered vaccines. Consideration should also be given to determining the size of residual cellular DNA as part of the validation process.

These tests may be conducted on the final product, in which case, the product should also conform to the specifications described here.

These tests may be omitted from routine testing, with the agreement of the national regulatory authority, if the manufacturing process is validated as consistently achieving the specification.
A.4.4 **Final bulk**

The final bulk should be aseptically prepared from one or more serotypes each derived from one or more virus pools obtained from substrates of which control cultures pass the tests specified in Section A.4.1. The virus concentration in the final formulation should be sufficient to ensure the dose which is consistent with that shown to be safe and effective in human clinical trials. The virus pools and final bulk should pass the tests specified in Sections A.4.3.3 and A.4.4.1.

The operations necessary for preparing the final bulk lot should be conducted in such a manner as to avoid contamination of the product.

In preparing the final bulk, any substances such as diluents or stabilizers that are added to the product should have been shown to the satisfaction of the national regulatory authority not to impair the safety and efficacy of the vaccine at the concentration used.

A.4.4.1 **Tests on the final bulk**

A44.1.1 **Sterility**

Each final bulk suspension should be tested for bacterial and fungal sterility according to Part A, sections 5.2 of the *General requirements for the sterility of biological substances* (16), or by a method approved by the national regulatory authority.

A.4.4.2 **Storage**

Until the bulk is filled into containers, the final bulk suspension should be stored under conditions shown by the manufacturer to allow it retain the desired biological activity.

A.5 **Filling and containers**

The requirements concerning filling and containers given in *Good manufacturing practices for biological products* (13) should apply to vaccine filled in the final form.

Care should be taken to ensure that the materials of which the container and, if applicable, transference devices and closure are made do not adversely affect the quality of vaccine and its diluent.

When a freeze-drying process is used for vaccine production, its validation should be submitted to the national regulatory authority for approval.

The manufacturers should provide the national regulatory authority with adequate data to prove the stability of the product under appropriate conditions of storage and shipping.
A.6  **Control tests on final lot**

Samples should be taken from each final lot for the following tests. Both freeze-dried vaccine and its diluent, if applicable, should be tested and should fulfil the requirements discussed in this section.

A.6.1  **Vaccine**

A.6.1.1  **Inspection of final containers**

Each container in each final lot should be inspected visually and those showing abnormalities should be discarded.

A.6.1.1.1  **Appearance**

The appearance of the freeze-dried or liquid vaccine should be described with respect to its form and colour. In the case of freeze-dried vaccines, a visual inspection should be performed of the freeze-dried vaccine, its diluent and the reconstituted vaccine. If reconstitution with the product diluent does not allow for the detection of particulates, an alternative diluent may be used.

A.6.1.2  **Identity**

The virus in one or more individually labelled final containers should be identified as rotavirus and, for multivalent vaccine formulations each serotype should be identified, by appropriate methods approved by the national regulatory authority. Methods such as plaque neutralization, and immunofocus assays in cell culture are suitable to identify the presence of rotavirus using rotavirus-specific polyclonal antiserum. PCR may also be appropriate, in this case the virus titration by quantitative PCR may serve as the identity test.

A.6.1.3  **Sterility**

Liquid or reconstituted vaccine should be tested for bacterial and fungal sterility according to the requirements in Part A, section 5.2 of the *General requirements for the sterility of biological substances* (16), or by the methods approved by the national regulatory authority.

A.6.1.4  **pH**

The pH of the final lot should be tested in a pool of final containers and an appropriate limit set to guarantee virus stability. In case of freeze-dried vaccines, pH should be measured after reconstitution of the vaccine with the diluent.

A.6.1.5  **Residual moisture**

The residual moisture in a representative sample of each freeze-dried lot should be determined by a method approved by the national regulatory authority and an appropriate limit set to ensure vaccine stability.
**A.6.1.6 Virus concentration**

The virus concentration in each of at least three final containers of the rotavirus vaccine final lot should be assayed individually for infectivity in a sensitive assay system in which interference or potentiation between the serotypes present in the vaccine does not occur.

An immunofocus or plaque assay may be used in MA-104, Vero or other sensitive cells to determine virus concentration. The assay is based on the visualization of infected areas of a cell monolayer directly or by probing with rotavirus serotype-specific monoclonal antibodies. Results should be recorded as FFU/ml or PFU/ml.

If immunological based assays are used, the specificity and lack of cross-reactivity of the antiserum must be verified. Alternatively, quantitative PCR detection of virus replication in a cell culture system may be used to provide an appropriate measure of infectivity. Results should be recorded as units of infectivity (UI/ml).

A cell culture infectious dose assay may also be used to determine virus concentration. Results should be recorded as CCID₅₀/ml.

The titre of each individual serotype should be determined and should fall within the specifications for potency. The assay method should include suitable qualified reference reagents for each serotype in the vaccine. The detailed procedures for carrying out the tests and for interpreting the results should be those approved by the national regulatory authority.

The national regulatory authority should approve a reference preparation of live attenuated rotavirus vaccine for use in tests to determine virus concentration.

Freeze-dried vaccine should be reconstituted with its diluent to determine virus concentration. A validated alternative diluent may be needed if the approved diluent is toxic to the cell cultures used in the assay. If a different diluent is used for this test, data to allow a comparison between the results with both diluents should be submitted for the approval of the national regulatory authority.

Internal consistency limits should be established by the manufacturer taking into account the vaccine dose shown to be safe and effective in human clinical trials. Specifications for virus concentration should essentially specify the minimum titre guaranteed to be contained in one human dose and this should be agreed with the national regulatory authority.

**A.6.1.7 Accelerated stability tests**

A representative number of the final containers should be exposed to an elevated temperature for a defined time period, using conditions based on
the manufacturer’s experience. The geometric mean of infectious virus titre of the containers that have been exposed should not have been decreased by more than a specific amount during the period of exposure. Estimation of the virus titre of non-exposed and exposed vials should be made in parallel and results expressed in terms of PFU, FFU, CCID\textsubscript{50} or UI per human dose. The maximum allowable loss of titre during the accelerated stability test should be confirmed on the basis of the manufacturer’s experience and approved by the national regulatory authority. For a multivalent vaccine, if there is no significant difference in the virus loss between serotypes, the loss may be based upon total virus concentration.

A.6.2 \textit{Diluents}

The requirements given in \textit{Good manufacturing practices for pharmaceutical products} (20) should apply for the manufacturing and control of diluents used to reconstitute live attenuated rotavirus vaccines and, if required, the antacid buffer used. An expiry date should be established for the diluent based upon stability data. If an antacid is to be used, the stability of the rotavirus in the presence of the antacid should be confirmed. For lot release of the diluent, tests for identity, appearance, pH, volume, sterility, and the content of key components should be done.

A.7 \textit{Records}

The requirements given in \textit{Good manufacturing practices for biological products} (13) should apply.

A.8 \textit{Samples}

The requirements given in \textit{Good manufacturing practices for biological products} (13) should apply.

A.9 \textit{Labelling}

The requirements given in \textit{Good manufacturing practices for biological products} (13) should apply, with the addition of the following.

The label on the carton enclosing one or more final containers, or the leaflet accompanying the container, should include the following information:

— a statement that the candidate vaccine fulfils Part A of these Guidelines;
— a statement of the nature of the preparation, specifying the designation of the strain(s) of rotavirus contained in the vaccine, the minimum amount of virus contained per human dose, the origin of the substrates used in the preparation of the vaccine and whether the vaccine strains were derived by molecular methods;
— the fact that the vaccine is not to be injected;
— a statement of the nature and quantity, or upper limit, of any antibiotic present in the vaccine;
— an indication that contact with disinfectants is to be avoided;
— a statement regarding the concomitant administration of rotavirus vaccine with other oral vaccines such as oral poliovirus vaccine and with other non-orally administered vaccines;
— a statement concerning administration to HIV-positive or other immunocompromised individuals;
— a statement indicating the volume and nature of the diluent to be added to reconstitute the vaccine, and specifying that the diluent to be used is that supplied by the manufacturer;
— a statement that after the vaccine is reconstituted, it should be used without delay, or if not used immediately, stored under defined conditions and in the dark for a maximum period defined by stability studies;
— a statement concerning storage conditions (temperature), expiry date, volume and instructions for reconstitution. Only one storage temperature and expiry date should be stated on the label and leaflet; and
— a statement indicating whether an antacid is to be given prior to or in combination with the vaccine at the time of vaccination.

It is desirable for the label or the leaflet to carry the names of both the producer and the source of the bulk material if the producer of the final vaccine did not prepare it.

A.10 **Distribution and shipping**

The requirements given in *Good manufacturing practices for biological products (13)* should apply.

Vaccine shipments should be maintained at the approved temperatures and parcels should contain cold-chain monitors.

For some products, freezing of the diluent should be avoided.

A.11 **Storage and expiry date**

The statements concerning storage temperature and expiry date of the vaccine and its diluent, if applicable, that appear on the label and in the leaflet, as recommended in *Good manufacturing practices for biological products (13)*, should be based on experimental evidence and should be submitted for approval to the national regulatory authority.

A.11.1 **Storage conditions**

Before being distributed by the manufacturing establishment or before being issued from a storage site, the vaccine should be stored at a temperature
shown by the manufacturer to be compatible with a minimal titre loss. The maximum duration of storage should be fixed with the approval of the national regulatory authority and should be such as to ensure that all quality specifications for final product including the minimum titre specified on the label of the container (or package) will still be maintained until the end of the shelf-life.

A.11.2 Expiry date

The expiry date should be defined on the basis of shelf-life and supported by the stability studies with the approval of the national regulatory authority and should relate to the date of the last satisfactory determination of virus concentration, performed in accordance with Part A, section A.4.3.3.5, i.e. the date on which the test system was inoculated.

The expiry dates for the vaccine and the diluent may be different.

Part B. Nonclinical evaluation of live attenuated rotavirus vaccines

Nonclinical evaluation of rotavirus vaccines should be based on existing WHO guidelines (1); however, the following rotavirus-specific issues should be considered.

In animal studies of rotavirus vaccines, oral dosing with antacid which corresponds to that intended for use in clinical trials is necessary; for example, in mice or in rats (using a strain of rat susceptible to human rotavirus infection) with the full human dose of vaccine. As rotavirus is not neurotropic, and neural tissue passage has not been used in the derivation of any of the vaccine strains, a neurovirulence test for each batch is not justified, nor is there a need to test the master or working seed lots. As with all live attenuated vaccines, special attention should be paid to evaluating the stability of attenuation phenotype by appropriate in vitro and in vivo assays. Nonclinical experimental studies to predict risks from excreted vaccine virus are not expected to be informative.

On the basis of a review of the literature, manufacturers should evaluate any risks to humans or animals if the vaccine virus is subsequently shown to be shed from vaccinees, taking into account the likelihood of excreted vaccine virus reassorting with wild-type viruses. Finally, the pathogenic mechanisms of intussusception associated with oral rotavirus vaccination are currently unknown and no suitable animal model is readily available to evaluate the risk of intussusception. Nevertheless, manufacturers should keep abreast of the evolving scientific knowledge and plan their strategy for preclinical vaccine evaluation accordingly.
Part C. Clinical evaluation of live attenuated rotavirus vaccines

The WHO guidelines on regulatory expectations for clinical evaluation are appropriate for development of rotavirus vaccines (2). Consideration should be given to the following rotavirus-specific issues.

C.1 Immune responses to rotavirus

Live attenuated rotavirus vaccines have been developed on the basis of evidence that natural rotavirus infections elicit protective immune responses, particularly against future severe rotavirus disease. For a period after rotavirus vaccination, much of the serum IgA is rotavirus-specific, so that rotavirus serum IgA levels act as a measure of seroconversion. Rotavirus serum IgA antibody responses have been used as measures of vaccine immunogenicity of all the candidate live attenuated rotavirus vaccines evaluated so far. Thus rotavirus serum IgA antibody responses should also be recorded for other vaccine candidates.

Nevertheless it should be noted that rotavirus serum IgA is not an immune correlate of protection for rotavirus vaccines. A role for neutralizing antibody in protective efficacy has been suggested, but the correlation between neutralizing antibody titres and clinical efficacy has not been demonstrated. These data have been recently reviewed for the different live attenuated rotavirus vaccine candidates (21).

C.2 Special considerations

There are additional specific issues that may affect the safety and efficacy of oral rotavirus vaccines. Factors which are considered important for evaluation pre-licensure include the following:

C.2.1 Vaccine virus shedding and transmission

Manufacturers should undertake studies to determine the amounts of the vaccine virus (if applicable, by serotype) shed by vaccinees and the duration of shedding. They should assess the transmissibility of vaccine strains to unvaccinated people during clinical studies of safety and efficacy. If vaccine virus is shed in sufficient quantities to make transmission feasible and/or if transmission from vaccines is demonstrated, then studies of the likelihood of reversion to wild type and the likelihood of the vaccine virus reassorting with wild-type rotaviruses are indicated.

C.2.2 Dose regimen

Clinical trials should be designed to determine the number of doses to be administered to elicit a measurable immune response and clinical efficacy.
Based on experience to date, at least two or three doses of live attenuated oral rotavirus vaccines are necessary to overcome interfering factors which may include maternal antibodies or concomitant administration of oral poliovirus vaccine (OPV).

C.2.3 Concomitant administration with routine childhood vaccines

As it is intended that live attenuated rotavirus vaccines would be incorporated into routine childhood immunization programmes, it is important to generate information on immune responses to co-administration with the routine childhood vaccines at the target ages of administration. In particular, co-administration with OPV should be studied, as both are live attenuated oral vaccines.

C.2.4 Vaccine safety

After licensure of a rhesus reassortant rotavirus vaccine, postmarketing surveillance in one country revealed an association with intussusception, a rare serious adverse event following administration of the vaccine to infants. This occurred in infants given the vaccine at an older age than that recommended by the manufacturer. In light of this experience, rotavirus vaccines must be assessed for any vaccine-attributable intussusception within 30 days after each dose of vaccine. However, even very large pre-licensure studies (for example in 70 000 infants) cannot rule out an association between vaccine and intussusception. They can only provide an estimate of the relative and absolute risk compared to placebo together with 95% confidence intervals that give an idea of the degree of risk that cannot be excluded. Such data are generated in clinical trials in selected populations and in which the doses of the regimen under study are controlled, so that the data may not predict risk in the post-licensure period. If post-licensure studies, currently in progress, reveal an age-related risk of vaccine-attributable intussusception, specifications may need to be considered for the age at first dose of vaccine (22).

In planning the size of future clinical safety trials for a new rotavirus vaccine, assumptions need to be made about the likely background incidence of intussusception. However, it may not be feasible to obtain accurate data on naturally occurring intussusception in all countries in which a study is to be performed. The calculation should state the degree of risk of vaccine-associated intussusception that the study should be able to assess. With appropriate justifications, sample sizes of < 70 000 for future pre-licensure clinical trials of new rotavirus vaccines may be acceptable.

C.2.5 Definition of clinical end-point in trials of protective efficacy

The results from clinical trials to date, show that rotavirus vaccines are more efficacious against severe illness than against mild or moderate disease. Standard definitions of a diarrhoea episode and the severity of illness
(recommended as the primary end-point of efficacy studies) are critical. Several methods for assessing the “severity” of rotavirus infection are available, for example, based on the scale described by Ruuska et al (23) and Clark et al (24). Hospitalization may not be appropriate as the only end-point because this is a context-sensitive situation which may not always reflect the severity of illness. However, hospitalization combined with grading of severity on an appropriate scale should give comparable results between study sites.

Usually the primary analysis is focused on prevention of acute rotavirus gastroenteritis due to the serotype(s) in the vaccine. It is acceptable that such primary analysis be based on those cases of acute rotavirus gastroenteritis that occur from 14 days onwards after administration of the last dose of the tested regimen. Consideration should also be given to sensitivity analysis to investigate efficacy against all cases of acute rotavirus gastroenteritis that occur from the time of the first dose onwards.

C.2.6 Other factors

Several other factors need to be considered when assessing new rotavirus vaccines, although some of these data may be generated post-licensure. Such data include those on use in special populations and in diverse geographical regions (developed or developing country settings), and interference by other health factors.

C.2.6.1 Vaccine safety in immunocompromised infants

No data on immunocompromised children are currently available. As with other live vaccines, live attenuated rotavirus vaccines should be shown to be safe in HIV-infected infants prior to general implementation in countries with high prevalence of HIV. Natural rotavirus infection has not been shown to be more serious or to be associated with greater safety risks in HIV-infected infants than in non-HIV-infected infants, although a slightly longer period of shedding of rotavirus was observed in one study.

C.2.6.2 Seasonality of rotavirus infection

Depending on where the pre-licensure efficacy data were generated (i.e. in areas with seasonal or year-round rotavirus infection), it may be appropriate to generate further data in alternative geographical areas. For example, in those settings with a marked seasonal pattern of rotavirus infection, the efficacy of rotavirus vaccine may be higher when it is administered shortly before the peak period for rotavirus circulation, possibly due to a booster effect of natural infection during the season. For this reason, it may be useful to assess efficacy in sequential seasons.

C.2.6.3 Diverse geographical and population settings

The efficacy and/or effectiveness of rotavirus vaccine needs to be studied in diverse geographical regions with different populations and different
socioeconomic conditions. Generally, data from clinical trials conducted in one part of the world would not necessarily be predictive of vaccine efficacy in other parts of the world (3). For example, clinical trials have shown variable levels of rotavirus-specific serum IgA with the same vaccine in populations in developed and less developed or developing countries. Furthermore, the assessment of protection provided by each serotype in the vaccine and/or against serotypes not in the vaccine (i.e. cross-protection) is inherently limited by the serotypes circulating in the countries where the study/studies was/were done during the observation period. Studies may lack the power to demonstrate efficacy by specific serotype. In these circumstances, data must be assessed on a case-by-case basis when considering any claims for vaccine serotype-specific and heterotypic protection.

Many factors are likely to be responsible for observed differences in immunogenicity and protective efficacy of the rotavirus vaccines in diverse geographical settings. In designing rotavirus vaccine trials (pre- or post-licensure), consideration should be given to controlling for the possible effects of factors such as the predominant circulating rotavirus serotypes, other vaccinations, malnutrition, vitamin and mineral deficiencies, concurrent enteric infections, malaria, parasitic infections, HIV, hepatitis, immunodeficiencies, maternally transmitted antibodies and breastfeeding.

C.2.6.4 Interchangeability

At present there are no data on safety, immunogenicity or efficacy when more than one vaccine type is used in one infant in the priming series. Although there may be difficulties in interpreting immunogenicity data in terms of predicting efficacy, and of complexities that would arise in studies of safety and efficacy when more than one vaccine type is given (e.g. a monovalent human rotavirus strain live attenuated vaccine followed by a multivalent human-bovine reassortant rotavirus strain live attenuated vaccine), such data would be welcome.

C.3 Postmarketing studies and surveillance

Large-scale postmarketing surveillance studies of safety and effectiveness are indicated once vaccines have been approved. In the case of rotavirus, knowledge of distribution of strains, establishment of country-specific laboratory expertise and education of physicians and policy-makers will be required to conduct these studies effectively. The aims of postmarketing surveillance of rotavirus vaccines may include evaluation of vaccine effectiveness in different populations and in settings in which more varied rotavirus serotypes predominate; the impact of emerging strains of rotavirus; monitoring the risk of vaccine-associated intussusception and of other potential adverse events; and evaluation of compliance with the recommended vaccination schedule. A standardized protocol for postmarketing surveillance is under development.
Part D. Guidelines for national regulatory authorities

D.1 General

The general recommendations for control laboratories given in the Guidelines for national authorities on quality assurance for biological products (25), which specify that no new biological substance should be released until consistency of manufacturing and quality as demonstrated by a consistent release of batches has been established, should apply.

The detailed production and control procedures and any significant changes made to them should be discussed with and approved by the national regulatory authority. The national regulatory authority should obtain the working reference from the manufacturers to establish a national working reference preparation for use until an international reference reagent becomes available.

D.2 Release and certification

A vaccine lot should be released only if it fulfills the national requirements and/or Part A of the present Guidelines. A protocol based on the model given 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 a request for release of vaccine for use.

A statement signed by the appropriate official of the national control laboratory should be provided if requested by a manufacturing establishment and should certify whether or not the lot of vaccine in question meets all national requirements, as well as Part A of these Guidelines. The certificate should also state the lot number, the number under which the lot was released, and the number appearing on the labels of the containers. In addition, the date of the last satisfactory determination of virus concentration as well as the expiry date assigned on the basis of shelf-life should be stated. A copy of the official national release document should be attached. The certificate should be based on the model given in Appendix 2.

The purpose of the certificate is to facilitate the exchange of live attenuated rotavirus vaccines between countries.

Authors

The first draft of these Guidelines (WHO/BS/05.2014) was prepared by Dr P.D. Minor, National Institute for Biological Standards and Control, Potters Bar, Herts, England; Dr M.L. Pombo, Instituto Nacional de Higiene Rafael Rangel, Control of Vaccines and Recombinant Department, Caracas, Venezuela; Dr W. Wainwright, Rotavirus Vaccine Program, PATH, Seattle, USA; with support from the WHO Secretariat (Dr D.J. Wood and Dr T.Q. Zhou, Quality Assurance and Safety of
A revised draft was prepared by Dr R. Dobbelaer, Biological Standardization, Scientific Institute of Public Health — Louis Pasteur, Belgium; Dr P.D. Minor, National Institute for Biological Standards and Control, Potters Bar, Herts, England; Dr M.L. Pombo, Instituto Nacional de Higiene Rafael Rangel, Control of Vaccines and Recombinant Department, Caracas, Venezuela; Dr W. Wainwright, Rotavirus Vaccine Program, PATH, Seattle, USA; Dr M. Powell, Medicines and Healthcare Products Regulatory Agency, London, England, with support from the WHO Secretariat (Dr D.J. Wood and Dr T.Q. Zhou, Quality Assurance and Safety of Biologicals/Immunization Vaccines and Biologicals/ Family and Community Health, WHO, Geneva, Switzerland; Dr M.P. Kieny and Dr D. Steele, Initiative for Vaccine Research/Immunization Vaccines and Biologicals/ Family and Community Health, WHO, Geneva, Switzerland; Dr A. Bentsi-Enchill, Vaccine Assessment and Monitoring/Immunization Vaccines and Biologicals/Family and Community Health, WHO, Geneva, Switzerland and Dr L. Chocarro, Access to Technologies/ Immunization Vaccines and Biologicals/Family and Community Health WHO, Geneva, Switzerland).

Acknowledgements

For the first draft of these guidelines, acknowledgements are due to the following participants at a WHO Informal Consultation on Quality, Safety and Efficacy Specifications for Live Attenuated Rotavirus Vaccines held in Mexico, DF, Mexico in February 2005:

- Dr C.D. Atreya, Center for Biologics Evaluation and Research, Food and Drug Administration, Rockville Pike, Bethesda, MD, USA; Dr L. Chocarro, Access to Technologies, World Health Organization, Geneva, Switzerland; Dr M. de los Angeles Cortes, Regional Advisor on Vaccines and Biologicals, Regional Office for the Americas, Pan American Health Organization, Washington, DC, USA; Dr R. Dobbelaer, Head, Biological Standardization, Scientific Institute of Public Health Louis Pasteur, Brussels, Belgium; Dr M. Franco, Instituto de Genetica Humana, Pontificia Universidad Javeriana, Bogotá, Colombia; Dr R. Glass, Chief, Viral Gastroenteritis Section, Centers for Disease Control and Prevention, Atlanta, GA, USA; Dr H. Greenberg, Senior Associate Dean for Research, Stanford University School of Medicine and the VA Palo Alto Health Care System, Stanford, USA; Dr C. Lecomte, Regulatory Affairs, Glaxo SmithKline Biologicals, Rixensart, Belgium; Dr A. MacLean, Director, Q Gen, Cooperative Research Centre for Vaccine Technology, Queensland Institute of Medical Research, Brisbane, Queensland, Australia; Dr P. Minor, Head, Division of Virology, National Institute for Biological Standards and Control, Potters Bar, Herts., England; Dr I. Perez-Schael, Chief of Section, Instituto de Biomedicina, Caracas, Venezuela; Dr M. Luz Pombo, Instituto Nacional de Higiene Rafael Rangel, Control of Vaccines and Recombinant Department, Caracas, Venezuela; Mr S. Prasad, Bharat Biotech International Ltd., Hyderabad, India; Dr L.P. Ruiz Jr., BIOVIRx Inc, Shoreview, MN, USA; Dr L. Saif, The Ohio State University, Ohio Agricultural Research and Development Center,
Food Animal Health Research Program, Wooster, OH, USA; Dr A. Shaw, Executive Director, Merck Vaccine Division, Merck and Co. Inc., West Point, USA; Dr L.S. Slamet, Deputy for Therapeutic Products, Narcotic, Psychotropic and Addictive Substance Control, Directorate General of Food and Drug Control, Ministry of Health, National Agency of Drug and Food Control, Jakarta Pusat, Indonesia; Dr D. Steele, Initiative for Vaccine Research, World Health Organization, Geneva, Switzerland; Dr G. Thiry, Director, Project Management for Research and Development, International AIDS Vaccine Initiative (IAVI), New York, NY, USA; Dr Timo Vesikari, University of Tampere Medical School, Tampere, Finland; Dr W. Wainwright, Rotavirus Vaccine Program, PATH (Seattle), Seattle, USA; Dr R.L. Ward, Division of Infectious Diseases, Children's Hospital Medical Center, Cincinnati, USA; and Dr D. Wood, Coordinator, Quality Assurance and Safety of Biologicals, World Health Organization, Geneva, Switzerland

Ma Cecilia Copello. National Regulatory Authority Argentina; Flavia Cardoso. National Regulatory Authority, Brazil; Patricia León Treviño. National Regulatory Authority, Colombia; Olga Jacobo Casanueva. National Regulatory Authority, Cuba; Ana Maria Jorquera. National Regulatory Authority, Chile; Ana Agaton, National Regulatory Authority, Venezuela; and Sonia Zamudio. National Regulatory Authority, México.

For the second draft, acknowledgements are due to the following participants at a WHO Informal Consultation on proposed WHO Guidelines to Assure the Quality, Safety and Efficacy of Live Attenuated Rotavirus Vaccines in Geneva, 29–30 August 2005:

Dr C.D. Atreya, Division of Viral products, Center for Biologics Evaluation and Research, Food and Drug Administration, Rockville, MD, USA; Dr R. Biellik, Rotavirus Vaccine Program (RVP), PATH-Europe, Ferney-Voltaire, France; Dr J. Bresee, Lead, Epidemiology Team, Respiratory and Enteric Virus Branch, Centers for Disease Control and Prevention, Rockville, MD, USA; Dr F. Cardoso de Melo, Head of Hemotherapeutic and Biological Products Unit, Agencia Nacional de Vigilancia Sanitaria, Ministerio da Saude, Brasilia, Brazil; Dr R.M. Dhere, Representative from Developing Country Vaccine Manufacturer’s Network (DCVMN), Director, Vaccine Production, Serum Institute of India Ltd, Maharashtra, India; Dr R. Dobbelaeer, Head, Biological Standardization, Scientific Institute of Public Health — Louis Pasteur, Brussels, Belgium; Dr Y. Lawanprasert, Food and Drug Administration, Ministry of Public Health, Thailand; Dr C. Lecomte, Representative from International Federation of Pharmaceutical Manufacturer’s Association (IFPMA), Technical Regulatory Affairs, GSK Biologicals, Belgium; Dr P. Minor, Head, Division of Virology, National Institute for Biological Standards and Control, Potters Bar, Herts, England; Dr S. Morgeaux, Agence Française de Sécurité Sanitaire des Produits de Santé, Direction des Laboratoires et des Contrôles, Unité de Contrôle des Médicaments Immunologiques, Lyon, France; Dr M. Luz Pombo, Instituto Nacional de Higiene Rafael Rangel, Control of Vaccines and Recombinant Department, Caracas, Venezuela; Dr Lucky S. Slamet, Deputy for Therapeutic Products, Narcotic, Psychotropic and Addictive Substance Control, National Agency of Drug and Food Control the Republic of Indonesia, Jakarta, Indonesia; Dr A.R. Shaw, Representative from International Federation of Pharmaceutical
Manufacturer’s Association (IFPMA), Executive Director, Merck Vaccine Division, West Point, Pennsylvania, USA; Dr J. Southern, Advisor to Medicines Control Council in South Africa, Ministry of Health, Cape Town, South Africa; Dr A. Tahlan, Joint Director and Government Analyst, Central Drugs Laboratory, Central Research Institute, Kasauli, India; Dr W. Wainwright, President, Biopharm Consulting Services, Director of Operations, BIOVIRx, Inc., Conestoga, PA, USA; Dr H. Yin, Director, Division of Biological Products, State Food and Drug Administration (SFDA), Beijing, People’s Republic of China; Dr Ma. Angeles Cortes, Regional Advisor on Vaccines and Biologicals, WHO Regional Office for the Americas/Pan American Sanitary Bureau, Washington, DC, USA; Dr J. Sokhey, WHO Regional Office for South-East Asia, World Health House, Indraprastha Estate, New Delhi, India; Dr D. Wood, Coordinator, Quality Assurance and Safety of Biologicals, World Health Organization, Geneva, Switzerland; Dr Tiequn Zhou, Scientist, Quality Assurance and Safety of Biologicals, World Health Organization, Geneva, Switzerland; Dr D. Steele, Scientist, Initiative for Vaccine Research, World Health Organization, Geneva, Switzerland; Dr L. Chocarro, Scientist, Access to Technologies, World Health Organization, Geneva, Switzerland; Dr A.D. Bentsi-Enchill, Medical Officer, Vaccine Assessment and Monitoring, World Health Organization, Geneva, Switzerland.

References


Appendix 1

Summary protocol of manufacturing and control of live attenuated rotavirus vaccines (oral)

The following protocol is intended for guidance, and indicates the information that should be provided as a minimum by the manufacturer to the national regulatory authority. The protocol must be accompanied by a lot release certificate from the licensing authority which may or may not be the country of manufacturing origin. Information and tests may be added or deleted as required by the national regulatory authority of the importing country, if applicable.

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 guidelines 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 guidelines in Part A of this document.

Summary information on the finished product (final lot)

International name: Live attenuated rotavirus vaccine (oral)
Commercial name: __________________________________________
Product licence (marketing authorization) number __________________
Country: ___________________________________________________
Name and address of manufacturer: ______________________________
Name and address of licence holder if different: ____________________

Final packing lot number: _____________________________________
Type of container: ___________________________________________
Number of containers in this packing lot: _________________________
Final container lot number: ___________________________________
Number of filled containers in this final lot: ______________________
Date of manufacture (filling or lyophilizing, if applicable): _________
Date on which last determination of virus concentration was started: _____________________________________
Shelf-life approved (months): __________________________________
Expiry date: ________________________________________________
Storage conditions: __________________________________________
Volume of single dose: ________________________________________
Volume of vaccine per container: _______________________________
Number of doses per container: _________________________________
Virus concentration per human dose:
Serotype: __________________________________________________
Serotype: __________________________________________________
Serotype: __________________________________________________
Serotype: __________________________________________________
Nature of any antibiotics present in vaccine and amount
per human dose: _____________________________________________
Production cell substrate: ____________________________________
Bulk No. of monovalent virus pools blended in multivalent
vaccine (if applicable): _______________________________________
Diluent or antacid (if applicable): _______________________________
  Lot number: _______________________________________________
  Date of manufacture: _______________________________________
  Expiry date: ______________________________________________

A genealogy of the lot numbers of all vaccine components used in the
formulation of the final product, diluent and antacid will be informative.
An example of a genealogy is given in Appendix 2.

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. If any cell lot, virus harvest or other intermediates intended
for production of the current lot was rejected, this should also be recorded
either in the following sections or on a separate sheet.

A.3  **Control of source materials**

The results of tests on the same master or manufacturing working cell
bank and the same virus master and manufacturing working seed lots are
submitted to and approved by the national regulatory authority during the
procedure for granting the licence or its variations and need not be re-
submitted at the time of lot release.

A.3.1 **Cell cultures for virus production**

Name and identification of cell substrate: __________________________
Origin and short history: ________________________________
Authority that approved cell bank: ____________________________
**Master cell bank (MCB)**

Lot number: ________________________________
Date MCB was established: ________________________
Date of approval by the national regulatory authority: ____________
Total number of ampoules stored: ________________________
Passage/population doubling level of MCB: ________________________
Maximum passage/population doubling level approved for MCB: ________________________
Storage conditions: ________________________________
Percentage of total MCB ampoules tested: ________________________
Identity test:
  Date of test ________________________________
  Method used ________________________________
  Results ________________________________
Results of tests for adventitious agents: ________________________
Results of tests for tumorigenicity (if applicable): ________________________
Tests for retroviruses (if applicable):
  Date of test ________________________________
  Method used ________________________________
  Results ________________________________

**Manufacturer's working cell bank (MWCB)**

Lot number: ________________________________
Date MWCB was established: ________________________
Total number of ampoules stored: ________________________
Passage/population doubling level of MWCB: ________________________
Maximum passage/population doubling level approved for MWCB: ________________________
Storage conditions: ________________________________
Percentage of total MWCB ampoules tested: ________________________
Identity test:
  Date of test ________________________________
  Method used ________________________________
  Results ________________________________
Results of tests for adventitious agents: ________________________
Results of tests for tumorigenicity (if applicable): ________________________
Tests for retroviruses (if applicable):
  Date of test ________________________________
  Method used ________________________________
  Results ________________________________

**Cell culture medium**

*Serum used in cell culture medium*

Animal origin of serum: ________________________________
Batch number: ____________________________
Vendor: ______________________________________
Country of origin: ____________________________
Certificate of freedom from TSE (yes/no): __________
Tests performed on serum: _______________________
  Methods used ________________________________
  Results ______________________________________

Trypsin used for preparation of cell cultures
Animal origin of trypsin: _________________________
Batch number _________________________________
Vendor: ______________________________________
Country of origin: ____________________________
Certificate of freedom from TSE (yes/no): __________
Tests performed on trypsin: ____________________
  Date of tests ________________________________
  Methods used ________________________________
  Results ______________________________________

A.3.2 Virus seeds
Virus strain(s) and serotype(s): ______________________
Substrate used for preparing seed lots: ________________
Origin and short history: __________________________
Authority that approved virus strain(s): ________________
Date approved: __________________________________

Virus master seed lot (VMS)
Lot number: ___________________________________
Date VMS was established: ______________________
Date approved by the National Regulatory Authority: __________
Total quantity of VMS stored: ____________________
Storage conditions: ______________________________
Passage level of VMS: ___________________________
Maximum passage level approved for VMS: __________

Manufacturer’s virus working seed lot (MVWS)
Lot number: ___________________________________
Date MVWS was established: _________________
Total quantity of MVWS stored: __________________
Storage conditions: ______________________________
Passage level of MVWS: _________________________
Maximum passage level approved for MVWS: __________
**Tests on virus master and working seed lots**

**Identity test:**
- Date of test ________________________________
- Method used ________________________________
- Results ________________________________

**Genotype/phenotype characterization:**
- Date of test ________________________________
- Method used ________________________________
- Results ________________________________

**Tests for bacteria, fungi and mycoplasmas**

**Tests for bacteria and fungi**

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Media used</th>
<th>Inoculum</th>
<th>Date test on</th>
<th>Date test off</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>20–25 °C</td>
<td>______</td>
<td>______</td>
<td>______</td>
<td>______</td>
<td>_____</td>
</tr>
<tr>
<td>30–36 °C</td>
<td>______</td>
<td>______</td>
<td>______</td>
<td>______</td>
<td>_____</td>
</tr>
<tr>
<td>Negative control</td>
<td>______</td>
<td>______</td>
<td>______</td>
<td>______</td>
<td>_____</td>
</tr>
</tbody>
</table>

**Tests for mycoplasmas**

**Standard culture method**

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Date test began</th>
<th>Date test ended</th>
<th>Incubation conditions</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solid media</td>
<td>______</td>
<td>______</td>
<td>______</td>
<td>_____</td>
</tr>
<tr>
<td>Liquid media</td>
<td>______</td>
<td>______</td>
<td>______</td>
<td>_____</td>
</tr>
<tr>
<td>Negative control</td>
<td>______</td>
<td>______</td>
<td>______</td>
<td>_____</td>
</tr>
<tr>
<td>Positive control cultures</td>
<td>______</td>
<td>______</td>
<td>______</td>
<td>_____</td>
</tr>
</tbody>
</table>

**Indicator cell-culture method**

<table>
<thead>
<tr>
<th>Cell substrate used ________________________________</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inoculum __________________________________________</td>
</tr>
<tr>
<td>Date of test ______________________________________</td>
</tr>
<tr>
<td>Passage number _____________________________________</td>
</tr>
<tr>
<td>Negative control ___________________________________</td>
</tr>
<tr>
<td>Positive controls ___________________________________</td>
</tr>
<tr>
<td>Date of staining ________________________________</td>
</tr>
<tr>
<td>Results __________________________________________</td>
</tr>
</tbody>
</table>

**Tests for adventitious viruses**

- Volume of virus seed samples for neutralization and testing ______
- Batch number(s) of antisera/antiserum used for neutralization of virus seeds ________________________________

**Tests in tissue cultures**

<table>
<thead>
<tr>
<th>Type of simian cells ________________________________</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quantity of neutralized sample inoculated __________________</td>
</tr>
<tr>
<td>Incubation conditions _______________________________</td>
</tr>
</tbody>
</table>
Date test began ____________________________  
Date test ended ____________________________  
Ratio of cultures viable at end of test ____________________________  
Results ____________________________  

Type of human cells ____________________________  
Quantity of neutralized sample inoculated ____________________________  
Incubation conditions ____________________________  
Date test began ____________________________  
Date test ended ____________________________  
Ratio of cultures viable at end of test ____________________________  
Results ____________________________  

Other cell types ____________________________  
Quantity of neutralized sample inoculated ____________________________  
Incubation conditions ____________________________  
Date test began ____________________________  
Date test ended ____________________________  
Ratio of cultures viable at end of test ____________________________  
Results ____________________________  

Tests in animals  

Test in adult mice  
Weight and number of animals ____________________________  
Routes and quantity of neutralized sample inoculated ____________________________  
Date test began ____________________________  
Date test ended ____________________________  
Ratio of animals surviving the observation period ____________________________  
Results ____________________________  

Test in suckling mice  
Age and number of animals ____________________________  
Routes and quantity of neutralized sample inoculated ____________________________  
Date test began ____________________________  
Date test ended ____________________________  
Ratio of animals surviving the observation period ____________________________  
Results ____________________________  

Test in guinea-pigs  
Weight and number of animals ____________________________  
Routes and quantity of neutralized sample inoculated ____________________________  
Date test began ____________________________  
Date test ended ____________________________  
Ratio of animals surviving the observation period ____________________________  
Results ____________________________
Additional tests ________________________________
Date of tests ________________________________
Methods used ________________________________
Results ________________________________

Virus concentration
Date of test ________________________________
Method used ________________________________
Results ________________________________

A.4 Control of vaccine production

Production cells
Lot number: ________________________________
Date of thawing ampoule of MWCB: ________________________________
Passage/population doubling level at virus inoculation: ______________
Maximum passage/population doubling level approved for vaccine production: ________________________________
Nature and concentration of antibiotics used in production cell culture maintenance medium: ________________________________
Identification and source of starting materials used in preparing production cells including excipients and preservative (particularly any materials of human or animal origin): ______________

A.4.1 Control of Cell Cultures

(Note: If more than one virus single harvest is used to produce a monovalent virus pool, then data on each lot of control cells should be provided.)

Amount or ratio of control cultures to production cell cultures: ________________________________

Incubation conditions: ________________________________

Period of observation of cultures: ________________________________
  Date started ________________________________
  Date ended ________________________________

Ratio of cultures discarded and reason: ________________________________

Results of observation: ________________________________

Date fluids collected: ________________________________

Date fluids pooled (if applicable): ________________________________

Tests for haemadsorbing viruses:
  Quantity of cells tested ________________________________
  Type of RBC used ________________________________
  Storage time and temperature of RBC ________________________________
  Incubation time and temperature of RBC ________________________________
Date test began __________________________________________
Date test ended __________________________________________
Results ________________________________________________

Additional tests if performed ___________________________________

Tests for other adventitious agents: 

**Test in production cells**

Date of sampling __________________________________________
Quantity of sample inoculated ______________________________
Incubation conditions _____________________________________
Date test began __________________________________________
Date test ended __________________________________________
Ratio of cultures viable at end of test _________________________
Uninoculated cell control __________________________________
Results ________________________________________________

**Test in human cells**

Type of human cells ________________________________________
Quantity of sample inoculated ______________________________
Incubation conditions _____________________________________
Date test began __________________________________________
Date test ended __________________________________________
Ratio of cultures viable at end of test _________________________
Uninoculated cell control __________________________________
Results ________________________________________________

**Test in other cell system**

Type of cells ______________________________________________
Quantity of sample inoculated ______________________________
Incubation conditions _____________________________________
Date test began __________________________________________
Date test ended __________________________________________
Ratio of cultures viable at end of test _________________________
Uninoculated cell control __________________________________
Results ________________________________________________

Identity test:

Date of test _____________________________________________
Method used ____________________________________________
Results ________________________________________________

A.4.2 **Cell cultures for vaccine production**

**Tests for adventitious agents**

Date of examination (inoculation) __________________________
Results ________________________________________________
**Tests for bacteria, fungi and mycoplasmas**

Date and volume of sampling ________________________________

Volume of samples tested ________________________________

### Tests for bacteria and fungi

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Media used</th>
<th>Inoculum</th>
<th>Date test on</th>
<th>Date test off</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>20–25 °C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30–36 °C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Tests for mycoplasmas

#### Standard culture method

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Date test on</th>
<th>Date test off</th>
<th>Incubation conditions</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liquid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### Indicator cell-culture method

<table>
<thead>
<tr>
<th>Cell substrate used</th>
<th>Date of test</th>
<th>Passage number</th>
<th>Negative control</th>
<th>Positive controls</th>
<th>Date of staining</th>
<th>Results</th>
</tr>
</thead>
</table>

### A.4.3 Control of single harvests

For multivalent vaccine, the following information for each virus serotype should be submitted.

If more than one single harvest is used to prepare a monovalent virus pool, the following information for each single harvest should be submitted.

#### Virus serotype

<table>
<thead>
<tr>
<th>Lot number of single harvest</th>
<th>Date of virus inoculation</th>
<th>Multiplicity of infection</th>
<th>Incubation conditions</th>
<th>Date of harvesting</th>
<th>Volume harvested</th>
<th>Date of sampling</th>
<th>Volume of sampling</th>
<th>Storage conditions and period</th>
</tr>
</thead>
</table>
**Monovalent virus pool (pre-clarification)**

Lot number of virus pool ________________________________
Date of pooling ________________________________
Virus single harvests pooled

<table>
<thead>
<tr>
<th>Lot number</th>
<th>Volume pooled</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Volume of virus pool after pooling ________________________________
Date of sampling ________________________________
Volume of sampling ________________________________
Storage of samples (if applicable) ________________________________

**Tests on single harvest or monovalent virus pools**

*(Tests may be done on individual single harvest or on the virus pools as approved by the national regulatory authority.)*

Identity

<table>
<thead>
<tr>
<th>Date of test</th>
<th>Method used</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Tests for bacteria, fungi and mycoplasmas

**Tests for bacteria and fungi**

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Media used</th>
<th>Inoculum</th>
<th>Date test began</th>
<th>Date test ended</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>20–25 °C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30–36 °C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Tests for mycoplasmas**

Standard culture method

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Date test began</th>
<th>Date test ended</th>
<th>Incubation conditions</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solid media</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liquid media</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive control cultures</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Indicator cell-culture method**

<table>
<thead>
<tr>
<th>Cell substrate used</th>
<th>Inoculum</th>
<th>Date of test</th>
<th>Passage number</th>
<th>Negative control</th>
<th>Positive controls</th>
<th>Date of staining</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results
**Tests for adventitious agents**

Volume of samples for neutralization and testing ___________________
Batch number(s) of antisera/antiserum used for neutralization _________

**Tests in tissue cultures**

- Type of simian cells ____________________________
- Quantity of neutralized sample inoculated ______________
- Incubation conditions _____________________________
- Date test began _________________________________
- Date test ended _________________________________
- Ratio of cultures viable at end of test ______________
- Results _________________________________________

- Type of human cells ____________________________
- Quantity of neutralized sample inoculated ______________
- Incubation conditions _____________________________
- Date test began _________________________________
- Date test ended _________________________________
- Ratio of cultures viable at end of test ______________
- Results _________________________________________

- Type of other cells ____________________________
- Quantity of neutralized sample inoculated ______________
- Incubation conditions _____________________________
- Date test began _________________________________
- Date test ended _________________________________
- Ratio of cultures viable at end of test ______________
- Results _________________________________________

<table>
<thead>
<tr>
<th>Cell substrate</th>
<th>Specification</th>
<th>Primary passage</th>
<th>Subculture passage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Test initiation</td>
<td>Test initiation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>date</td>
<td>date</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No. of flasks</td>
<td>No. of flasks</td>
</tr>
<tr>
<td></td>
<td></td>
<td>tested</td>
<td>tested</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Results</td>
<td>Results</td>
</tr>
</tbody>
</table>

- Cytopathic effect
- Haemadsorption
- Positive control virus
- Negative control

**Virus concentration**

- Date of test _____________________________
- Method used _____________________________
- Results __________________________________
Tests for consistency of virus characteristics
(Tests are performed during vaccine development and process validation,
may not be required for batch release.)

<table>
<thead>
<tr>
<th>Item tested</th>
<th>Date of test</th>
<th>Methods used</th>
<th>Results</th>
</tr>
</thead>
</table>

Storage conditions and period

Control of clarified monovalent virus pool

<table>
<thead>
<tr>
<th>Lot number of monovalent virus pool</th>
<th>Date of clarification</th>
<th>Methods used for clarification</th>
<th>Volume of virus pool before clarification</th>
<th>Volume of virus pool after clarification</th>
<th>Date of sampling</th>
<th>Volume of sampling</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Sterility</th>
<th>Specification</th>
<th>Date test initiated</th>
<th>Method</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus concentration</td>
<td>___________</td>
<td>___________</td>
<td>_________</td>
<td>_________</td>
</tr>
<tr>
<td>Tests for residual cellular DNA</td>
<td>___________</td>
<td>___________</td>
<td>_________</td>
<td>_________</td>
</tr>
</tbody>
</table>

A.4.4 Final bulk

<table>
<thead>
<tr>
<th>Lot number</th>
<th>Date of formulation</th>
<th>Total volume of final bulk formulated</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Lot number</th>
<th>Volume added</th>
<th>Virus concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>______</td>
<td>__________</td>
<td>___________</td>
<td>__________</td>
</tr>
<tr>
<td>______</td>
<td>__________</td>
<td>___________</td>
<td>__________</td>
</tr>
<tr>
<td>______</td>
<td>__________</td>
<td>___________</td>
<td>__________</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name</th>
<th>Lot number</th>
<th>Volume added</th>
</tr>
</thead>
<tbody>
<tr>
<td>______</td>
<td>__________</td>
<td>__________</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sterility</th>
<th>Specification</th>
<th>Date test initiated</th>
<th>Method</th>
<th>Results</th>
</tr>
</thead>
</table>

Storage conditions and period
Approved storage period

A.5 **Filling and containers**

**Lot number**

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

A.6 **Control tests on final lot**

A.6.1 **Vaccine**

*Inspection of final containers*

Appearance

Date of test

Results

Before reconstitution

After reconstitution

Diluent used

Lot number of diluent used

*Identity*

Date test began and ended

Method used

Results

Lot number of reference reagents

<table>
<thead>
<tr>
<th>Specification</th>
<th>Date test initiated</th>
<th>Method</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterility</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Diluent used

Lot number of diluent used

*PH*

Date of test

Method used

Results

<table>
<thead>
<tr>
<th>Specification</th>
<th>Date test initiated</th>
<th>Method</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>PH</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Diluent used**
Lot number of diluent used

**Residual moisture (if applicable)**
Date of test
Method used
Results

**Virus concentration**
Date titration began and ended
Method used for titration
Results

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Virus titre</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Lot number of reference virus
Lot number of other reference reagents if used
Diluent used
Lot number of diluent used

**Accelerated stability tests**
Duration of exposure
Temperature of exposure
Date titration began and ended
Method used for titration
Results

**Total virus titre**

Exposed sample
Non-exposed sample
Titre reduction

Lot number of reference virus
Lot number of other reference reagents if used
Diluent used
Lot number of diluent used

**A.6.2 Diluents**
Nature and volume
Lot number
Date of manufacture
Storage conditions and period
Expiry date
<table>
<thead>
<tr>
<th>Specification</th>
<th>Date test initiated</th>
<th>Method</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterility</td>
<td>___________</td>
<td>_______</td>
<td>_______</td>
</tr>
<tr>
<td>Identity</td>
<td>___________</td>
<td>_______</td>
<td>_______</td>
</tr>
<tr>
<td>pH</td>
<td>___________</td>
<td>_______</td>
<td>_______</td>
</tr>
<tr>
<td>Physical inspection</td>
<td>___________________________</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Content of key components:</td>
<td>___________________________</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>___________________________</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>___________________________</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Certification by the manufacturer**

Name of head of production (typed) ________________________________

*Certification by the person from the control laboratory of the manufacturing company taking over responsibility for the production and control of the vaccine:*

I certify that lot no. ____________ of live attenuated rotavirus vaccine (oral), whose number appears on the label of the final container, meets all national requirements and satisfies Part A of the Requirements for Biological Substances No. ___________ of live attenuated rotavirus vaccines (oral).

Signature: ____________________________________________________

Name (typed): ________________________________________________

Date: ______________________________________________________
Appendix 2
Genealogy of vaccine production process

- Virus Master Seed
  - Type 1
  - Lot no.
- Virus Working Seed
  - Type 1
  - Lot no.
- Single Harvest
  - Type 1
  - Lot no.
- Single Harvest
  - Type 1
  - Lot no. 2
- Single Harvest
  - Type 1
  - Lot no.
- Monovalent Virus Pool
  - Type 1
  - Lot no.
- Clarified Monovalent Virus Pool
  - Type 1
  - Lot no.
- Virus Master Seed
  - Type 2
  - Lot no.
- Virus Working Seed
  - Type 2
  - Lot no.
- Single Harvest
  - Type 2
  - Lot no.
- Single Harvest
  - Type 2
  - Lot no. 2
- Single Harvest
  - Type 2
  - Lot no.
- Monovalent Virus Pool
  - Type 2
  - Lot no.
- Clarified Monovalent Virus Pool
  - Type 2
  - Lot no.
- Virus Master Seed
  - Type ...
  - Lot no.
- Virus Working Seed
  - Type ...
  - Lot no.
- Single Harvest
  - Type ...
  - Lot no.
- Single Harvest
  - Type ...
  - Lot no. 2
- Single Harvest
  - Type ...
  - Lot no.
- Monovalent Virus Pool
  - Type ...
  - Lot no.
- Clarified Monovalent Virus Pool
  - Type ...
  - Lot no.
Genealogy of final bulk lot and final lot
Appendix 3

Model certificate for the release of live attenuated rotavirus vaccines

This certificate is to be provided by the national regulatory authority of the country where the vaccines have been manufactured, on request by the manufacturer
Certificate no. _______________

Lot release certificate

The following lot(s) of live attenuated rotavirus vaccine (oral) produced by ____________________1 in _______________2 whose numbers appear on the labels of the final containers, meet all national requirements3 and Parts A4, B and C of Guidelines for Live Attenuated Rotavirus Vaccine (oral), (2007)5 and comply with Good Manufacturing Practices for pharmaceutical products6 and Good manufacturing practices for biological products.7

As a minimum, this certificate is based on examination of the summary protocol of manufacturing and control.

<table>
<thead>
<tr>
<th>Final lot no.</th>
<th>Number 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>

The director of the national regulatory authority (or authority as appropriate):
Name (typed) ____________________________________________
Signature _____________________________________________
Date _________________________________________________

---

1 Name of manufacturer.
2 Country of origin.
3 If any national requirements are not met, specify which one(s) and indicate why release of the lot(s) has nevertheless been authorized by the national regulatory authority.
4 With the exception of provisions on distribution and shipping, which the national regulatory authority may not be in a position to assess.
Annex 4

Recommendations for the production, control and regulation of human plasma for fractionation

1 Introduction

2 International Biological Reference Preparations

3 Glossary

4 General considerations
   4.1 Range of products made from human blood and plasma
   4.2 Composition of human plasma
   4.3 Pathogens present in blood and plasma

5 Measures to exclude infectious donations
   5.1 Appropriate selection of blood/plasma donors
   5.2 Screening of blood/plasma donations for infectious markers
   5.3 Epidemiological surveillance of donor population
   5.4 Strict adherence to Good Manufacturing Practices
   5.5 Post-donation events

6 Production of plasma for fractionation
   6.1 Methods used to obtain plasma for fractionation
   6.2 Characteristics of plasma for fractionation
   6.3 Premises and devices for collection of plasma for fractionation
   6.4 Blood/plasma collection process
   6.5 Separation of plasma
   6.6 Freezing of plasma
   6.7 Storage of plasma
   6.8 Compliance with plasma fractionator requirements
   6.9 Release of plasma for fractionation
   6.10 Packaging of plasma
   6.11 Transportation of plasma
   6.12 Recall system

7 Quality assurance system and Good Manufacturing Practices
   7.1 Organisation and personnel
   7.2 Documentation system
   7.3 Premises and equipment
   7.4 Materials
   7.5 Validation programme
7.6 Quality monitoring data
7.7 Virology safety testing
7.8 Electronic information system
7.9 Storage and transport
7.10 Change control system
7.11 Quality assurance auditing
7.12 Defect reporting system
7.13 Quality agreement between blood establishment and fractionator
7.14 Blood/plasma establishment audit and inspection

8 Regulatory control of plasma for fractionation
8.1 Role of national regulatory authority
8.2 Establishment licence and inspections
8.3 Impact of Good Manufacturing Practices
8.4 Inspections

Authors

References

Appendix 1
Plasma products and clinical applications

Appendix 2
Donor selection

Appendix 3
Donor immunization and plasmapheresis for specific immunoglobulins

Appendix 4
Contract plasma fractionation programme

Appendix 5
Technical points to consider in establishing plasma specifications criteria and obligations between blood establishment and plasma fractionator
## Contents

### Introduction
- Opening remarks by Secretary of the Expert Committee 2

### General
- Developments in biological standardization 2
- WHO programmatic issues 2
- Vaccines and biological therapeutic products 4
- Maintenance of capacity for production of MCR-5 human diploid fibroblast cells 6
- Blood products and related in vitro diagnostic devices (IVDs) 8
- Advancement of technical expertise of regulatory authorities in the area of blood products and IVDs 10
- Quality, safety and efficacy of animal plasma-derived antisera 10
- Global needs in standardization of products derived by biotechnology 12
- International Non-proprietary Names for gene therapy products 13
- Reports from the WHO International Laboratories and WHO Collaborating Centres 14
- Feedback from users of WHO biological standardization products 19

### International guidelines, recommendations and other matters related to the manufacture and quality control of biologicals
- Guidelines for assuring the quality and nonclinical safety evaluation of DNA vaccines 20
- Recommendations for rabies vaccines 21
- Guidelines to assure the quality, safety and efficacy of live attenuated rotavirus vaccines (oral) 22
- WHO Biosafety guidelines for the production and quality control of human influenza pandemic vaccine strains 23
- Recommendations for whole cell pertussis vaccines 24
- Recommendations for the production, control and regulation of human plasma for fractionation 25
- WHO guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products 25
- Guidelines to assure the quality, safety and efficacy of human cells and tissues for transplantation 27
- Guidelines for good manufacturing practice for biological products 29
- Guidelines for good manufacturing practice for blood establishments 30
- Stability of vaccines 31
- Flavivirus vaccines — regulatory expectations 32
- Guidelines for acellular pertussis vaccines 33
- Recommendations for bacille Calmette-Guérin vaccines 34

### International reference materials
- Proposals for discontinuation of reference preparations 35
Antigens and related substances

Yellow fever vaccine — minimum specifications in International Units per 0.5 ml dose
Smallpox vaccine — stability studies
Poliovirus, Sabin type 3 — neurovirulence test reference
*Haemophilus influenzae* type b capsular polysaccharide

Antisera

Dengue virus antibody, human serum
Japanese encephalitis virus, human serum
Anti-human platelet antigen-1a

Blood products and related substances

World Health Organization/International Society of Thrombosis and Haemostasis Liaison Committee report
Anti-A and anti-B blood grouping reagents
Prothrombin mutation
Vitamin B12 and folate in human serum
Blood coagulation factor V (plasma) human
Blood coagulation factor XI (plasma) human
Thromboplastin, rabbit, plain

Cytokines, growth factors and endocrinological substances

Vascular endothelial growth factor
Keratinocyte growth factor
Measurement of relative potencies of thermal degradation samples of the WHO international standard of interferon alpha 2b

Diagnostic reagents

Reference materials for in vitro diagnostic devices
HIV-1 RNA nucleic acid amplification test
Anti-HIV tests

Annex 1
Guidelines for assuring the quality and non-clinical safety evaluation of DNA vaccines

Annex 2
Recommendations for inactivated rabies vaccine for human use produced in cell substrates and embryonated eggs

Annex 3
Guidelines to assure the quality, safety and efficacy of live attenuated rotavirus vaccines (oral)

Annex 4
Recommendations for the production, control and regulation of human plasma for fractionation
Annex 5
WHO biosafety risk assessment and guidelines for the production and quality control of human influenza pandemic vaccines 265

Annex 6
Recommendations for whole cell pertussis vaccine 301

Annex 7
Biological Substances: international standards and reference reagents 335

Annex 8
Recommendations, guidelines and other documents for biological substances used in medicine 337
Members

Dr W.G. van Aken, Amstelveen, the Netherlands

Dr R. Dobbelaer, Head, Biological Standardization, Scientific Institute of Public Health — Louis Pasteur, Brussels, Belgium (Chairman)

Dr F. Fuchs, Director — Lyon Site, French Agency for Safety of Health Products, Lyon, France

Dr V. Grachev, Deputy Director, MP Chumakov Institute of Poliomyelitis and Viral Encephalitides, Moscow, Russian Federation

Dr A. Homma, Director, Bio-Manguinhos, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil

Dr T. Kurata, Director General, National Institute of Infectious Diseases, Tokyo, Japan

Dr J. Löwer, Director, Paul Ehrlich Institut, Langen, Germany

Dr P. Minor, Head, Division of Virology, National Institute for Biological Standards and Control, Potters Bar, Herts., England

Dr F. Reigel, Head, Swissmedic, Biological Medicines and Laboratories, Agency for Therapeutic Products, Berne, Switzerland (Rapporteur)

Professor G.N. Vyas, Department of Laboratory Medicine, University of California, San Francisco, California, USA (Vice-Chairman)

Representatives of other organizations

Council of Europe, European Directorate for the Quality of Medicines
Mr J.-M. Spieser, European Directorate for the Quality of Medicines, European Pharmacopoeia Commission, Strasbourg, France

Dr K. H. Buchheit, European Directorate for the Quality of Medicines, European Pharmacopoeia Commission, Strasbourg, France

Developing Country Vaccine Manufacturer’s Network
Dr S. Jadhav, Executive Director, Serum Institute of India Ltd, Pune, India

European Diagnostic Manufacturers Association
Dr H. Bayer, Roche Diagnostics, Mannheim, Germany

Eye Bank Association of America
P. Dahl, Executive Director/CEO, The Eye-Bank for Sight Restoration, Inc New York, USA

International Association of Biologicals
Dr A. Eshkol, Vice-President, Scientific Affairs, Serono International SA, Plan-Les-Ouates, Switzerland

International Federation of Clinical Chemistry and Laboratory Medicine
Professor J.-C. Forest, Centre Hospitalier Universitaire de Québec, Québec, Canada
International Federation of Pharmaceutical Manufacturers and Associations
Dr A. Sabouraud, Responsible Pharmacist, Quality Control of Development Products, Sanofi Pasteur SA, Marcy l’Etoile, France
Dr M. Düchene, Head of Product Life Cycle Management, GlaxoSmithKline Biologicals, Rixensart, Belgium

International Society of Blood Transfusion/European Plasma Fractionation Association
Dr P. Strengers, Amsterdam, the Netherlands

International Society on Thrombosis and Haemostasis
Professor I. Peake, Deputy Director, Division of Genomic Medicine, University of Sheffield, Royal Hallamshire Hospital, Sheffield, England

Plasma Protein Therapeutics Association
Dr R. Büchel, Director PPTA Source/European Plasma Collection Committee, Brussels, Belgium

United States Pharmacopeia
Dr R. Dabbah, Division Department of Standards Development US Pharmacopeia, Rockville, MD, USA
Secretariat

Dr Y. Arakawa, Director, Department of Bacterial Pathogenesis and Infection Control, National Institute of Infectious Disease, Tokyo, Japan (Temporary Adviser)

Dr T. Barrowcliffe, Head, Haematology, National Institute for Biological Standards and Control, Potters Bar, Herts., England (Temporary Adviser)

Dr A. Bristow, Head, Technology Development and Infrastructure, National Institute for Biological Standards and Control, Potters Bar, Herts., England (Temporary Adviser)

Dr T. Burnouf, Human Plasma Product Services, Lille, France (Temporary Adviser)

Dr D. Calam, Pewsey, Wilts., England (Temporary Adviser)

Dr F. Cardoso de Melo, Head of Hemotherapic and Biological Products Unit, Agencia Nacional de Vigilancia Sanitaria, Ministerio da Saude, Brasilia DF, Brazil (Temporary Adviser)

Dr M. Corbel, Division of Bacteriology, National Institute for Biological Standards and Control, Potters Bar, Herts. England (Temporary Adviser)

Dr P. Dubord, University of British Columbia, Vancouver BC, Canada (Temporary Adviser)

Dr M. Ferguson, National Institute for Biological Standards and Control, Potters Bar, Herts. England (Temporary Adviser)

Mrs D. Fehily, Como, Italy (Temporary Adviser)

Dr E. Griffiths, Associate Director General, Biologics and Genetic Therapies, Health Canada, Ottawa, Ontario, Canada (Temporary Adviser)

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

Mrs T. Jivapaisarnpong, Director, Division of Biological Products, Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand (Temporary Adviser)

Dr V.K. Kashyap, Director, National Institute of Biologicals, Ministry of Health and Family Welfare, Noida, India (Temporary Adviser)

Dr H. Klein, National Institutes of Health, Warren G. Magnuson Clinical Center, Department of Transfusion Medicine, Bethesda, MD, USA (Temporary Adviser)

Dr R.G. Kreeftenberg, Product Manager, Netherlands Vaccine Institute, Bilthoven, Netherlands (Temporary Adviser)

Dr M. Luz Pombo, Chief, Department Control of Vaccines and Recombinant Products, Instituto Nacional de Higiene “Rafael Rangel”, Caracas, Venezuela (Temporary Adviser)

Dr M. Nubling, Paul Ehrlich Institut, Langen, Germany (Temporary Adviser)

Dr S.-N. Park, Director, Division of Viral Vaccines, Korea Food and Drug Administration, Seoul, Republic of Korea (Temporary Adviser)

Dr M. Powell, Medicines and Healthcare Products Regulatory Agency, London, England (Temporary Adviser)
Dr C. Schärer, Swissmedic, Swiss Agency for Therapeutic Products, Inspectorates/Biologicals, Berne, Switzerland (Temporary Adviser)

Dr J.A. Southern, Advisor to Medicines Control Council in South Africa, Simonstown, South Africa (Temporary Adviser)

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

Dr Y. Vajaradul, The Bangkok Biomaterial Center, Siriraj Hospital, Mahidol University, Bangkok, Thailand (Temporary Adviser)

Dr J. Weir, Acting Deputy Director, Office of Vaccines Research and Review, Center for Biologics Evaluation and Research, Food and Drug Administration, Rockville, MD, USA (Temporary Adviser)

Dr D. Wood, Coordinator, Quality Assurance and Safety of Biologicals, World Health Organization, Geneva, Switzerland (Secretary)

Dr J. Wood, National Institute for Biological Standards and Control, Potters Bar, Herts. England (Temporary Adviser)

Professor Hongzhang Yin, Division of Biological Products, State Food and Drug Administration, Beijing, People’s Republic of China (Temporary Adviser)
Introduction

The WHO Expert Committee on Biological Standardization met in Geneva from 24 to 28 October 2005. The meeting was opened by Dr J.M. Okwo-bele, Director, Department of Immunization, Vaccines and Biologicals on behalf of the Director-General.

Dr Okwo-bele emphasized the importance of the work of the Committee, which as one of the longest standing WHO Committees started its work as early as 1947. He stressed the importance of its work in developing, establishing and promoting written technical standards to assure the quality, safety and efficacy of vaccines, biological therapeutic products, blood products and selected in vitro diagnostic devices (IVDs). He also noted that WHO biological reference preparations are important tools that allow the comparability of data worldwide in diverse fields of medical practice.

Dr Okwo-bele summarized the key policy developments in biological standardization since the Committee had last met. He highlighted high level discussions between WHO and the European Union (EU) Commission that led to the acceptance by the EU of the principle that WHO biological reference materials could be regarded as “higher order” reference materials in the context of EU IVD legislation. This decision was based on the example provided by the 2nd International Standard for Hepatitis B surface antigen, established by the Committee in 2004.

He also reported that, in response to the need of the Secretariat and the advice from the Committee, the meetings of the Expert Committee on Biological Standardization would be run in a new way. The first and last days would be plenary sessions, whereas the middle three days would consist of two parallel tracks, one for vaccines and biological therapeutic products and the other for blood products and related IVDs.

Dr Okwo-bele informed the Committee that new experts had been appointed to the Expert Advisory Panel on Biological Standardization. This would strengthen support to WHO by increasing the diversity of subject experts, and the geographic and gender balance of the Committee. He also highlighted key public health issues that would be considered by the Committee during its meeting. This included establishment of technical documents for the safe production of pandemic influenza vaccines; guidance on regulatory expectations for live attenuated rotavirus vaccines; concerns about the transmission of variant Creutzfeldt–Jakob disease (vCJD) through blood transfusion; and, the safety of products from human plasma. Dr Okwo-bele reminded the participants at the meeting that only 60% of blood products worldwide were produced under stringent regulatory oversight. The new document on plasma for fractionation would be expected to contribute significantly to improving the safety of patients.
Dr Okwo-bele reminded the members of the Committee that they do not represent organizations or governments, but had been invited by WHO because of their individual expertise. He reminded the Committee that all decisions made should be based on sound scientific principles. Finally he invited all participants in the meeting to actively contribute in their respective capacities.

Dr Roland Dobbelaer was elected as Chairman of the meeting, Dr Girash Vyas, Vice-Chairman and Dr Franz Reigel, as Rapporteur. After all the participants had been introduced, the Committee adopted the agenda (WHO/BS.04.2005) and the timetable proposed.

Opening remarks by Secretary of the Expert Committee

Dr David Wood welcomed the participants. In order to manage the two parallel sessions for the middle days of the meeting, Dr Johannes Löwer was elected as Chairman, Professor G.N. Vyas as Vice-chairman and Dr Franz Reigel as Rapporteur for the track for blood products and Dr Roland Dobbelaer as Chairman, Dr Takeshi Kurata as Vice-Chairman and Dr Morag Ferguson as Rapporteur for the track of vaccines and biological therapeutic products.

General

Developments in biological standardization

WHO programmatic issues

Dr David Wood recalled the WHO mandate of its 192 Member States to develop, establish and promote international standards for biological products. In operational terms this mandate covers vaccines, biological therapeutic products, blood products and selected IVDs.

He discussed recent organizational changes that are intended to provide clear strategic directions, an appropriate organizational structure, and appropriate financial and human resources to fulfil the ambitions of WHO. The new (11th) WHO General Programme of Work proposes a global health agenda for the years 2006–2015, and identifies among the key challenges to health the need to close gaps in implementation of knowledge. The Organization is also emphasizing a results-based management system using performance indicators. The Committee stressed that WHO should ensure that biological standardization activities remain highly integrated whatever the organizational location of the unit(s) that perform the activities.

Dr Wood presented a process for establishing priorities for written standards for public health issues of global importance and also the establishment of
a portfolio of proposed new and replacement reference preparations. He described a review process for the draft priorities by stakeholders including validation by the Expert Advisory Panel for Biological Standardization and the Committee. The Committee agreed that it is essential to continue to support the research base to develop standards so that the WHO biological standardization process produces relevant standards that contribute to global harmonization. Moreover WHO aims to develop strategies to assess the impact of WHO standards, to strengthen the global capacity to regulate biologicals and, in the long term, to ensure that only biological products of assured quality are used in health systems.

WHO aims to foster a broader geographical representation for its collaborating centres, and to strengthen the relationships between these centres. With regard to the biological reference materials programme, Dr Wood reported some recent developments such as transfer of custodianship of antibiotic standards from the National Institute for Biological Standards and Control, England to the European Directorate for the Quality of Medicines (EDQM) in France; progress with development of regional working reference materials (an initiative being implemented by the WHO Regional Office for South-East Asia); a newly established working group on reference material stability; progress in capacity building in biological standardization (such as training in biological standardization being provided by the collaborating centres); and new categories of reference materials (such as genetic testing standards, and a proposal to consider standards for cell therapy reference materials). The Committee strongly supports the concept of networking between the laboratories that operationalize the WHO biological standards programme.

Promotion of WHO biological standards will be improved through a variety of means such as publications and web sites, e-learning materials, interactions with regulators, for example via the recently established Developing Countries Vaccines Regulators Network and the proposed network of blood products regulators. New members are being actively recruited to strengthen the expertise and the geographical and gender balance of the Expert Advisory Panel (EAP). A web-based collaborative workspace has been established which provides the possibility for interactive consultations, and had been used, for example, to consult the EAP on the agenda for the present meeting. The web space will be increasingly used to consult the EAP on policy and strategy issues as well as scientific matters. It will help to develop a collective sense of community and improve knowledge sharing. Contacts with other users will be improved to promote WHO standards and to receive input on needs and concerns.

In summary, WHO’s biological standardization programme will provide global standards for biological medicines, promote and implement global
norms and standards for biological medicines, and continue to strive to provide the most relevant products to improve global health.

**Vaccines and biological therapeutics**

Dr David Wood outlined recent developments in immunization, changes at WHO, recent activities to support the science base for norms and standards, and short-term priorities.

The new Global Immunization Vision and Strategy 2006–2015 provides an ambitious framework for immunization for the next decade and will contribute to improved global health through expanding the scope of immunization activities in many countries. This will include introduction of new vaccines for children, reaching older age groups with vaccines, using vaccines in epidemic preparedness and control and also using vaccines to prevent some types of cancer. Many new vaccines currently under development have the potential to prevent childhood deaths (for example vaccines against rotavirus, measles, pertussis, tetanus, yellow fever, malaria, human immunodeficiency virus (HIV), tuberculosis (TB), meningococcal A/C diseases, Japanese encephalitis and streptococcal pneumonia). Dr Wood emphasized that new regulatory paradigms were emerging. For example, some vaccines were being registered first in the countries with the higher disease burdens rather than in the developed world. This placed large responsibilities on the regulators in these countries and emphasized the need for continually updated, or new, WHO guidelines and recommendations.

Dr Wood gave an overview of the background and processes of the new strategic direction and competency review at WHO. The core competences at WHO headquarters in the IVB Department were in the Innovation Unit (IVR Unit), Quality, Safety and Standards (QSS Team), and Access (EPI+ Team). Biological standardization activities were to be managed by the QSS team. The impact of this change would be to strengthen norms and standards activities in the vaccine field, and represented a strong commitment of the Organization.

Dr Wood summarized some important recent scientific activities, namely a meeting held in April 2005 on the application of molecular methods in the control of vaccines, that included a review of safety issues associated with residual cellular DNA in vaccines, and an informal consultation on the scientific bases for regulatory evaluation of candidate human vaccines manufactured in plants, that occurred in January 2005. The reports of both meetings had been submitted for publication in peer reviewed journals. He also highlighted a WHO/International Association for Biologicals/EDQM workshop on neurovirulence tests for live virus vaccines held in January 2005, the report of which was submitted for publication in a peer-reviewed journal.
Dr Wood presented the short-term priorities in developing norms and standards in the vaccines area and invited comments from the Committee. The agreed written standards and Working Group activities are given in Table 1. He also informed the Committee of the establishment of indicators for the performance of the biological standards programme. For the period 2006–2007 these are listed in Table 2.

**Table 1**

**Short-term priorities for vaccines and biological therapeutics**

<table>
<thead>
<tr>
<th>Document</th>
<th>Justification for assignment as a priority</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1a. Written standards (to specify production, QC, nonclinical and clinical trial standards) to be established by the ECBS in 2006–2007</strong></td>
<td></td>
</tr>
<tr>
<td>Proposed revised standards</td>
<td></td>
</tr>
</tbody>
</table>
| OPV (TRS 904, 2002) | 1. Inclusion of biosafety specifications for OPV stockpile production in post-OPV era  
2. Elimination of quality control need for wild polio type 1 |
| IPV (TRS 910, 2002) | 1. Update of biosafety specifications for production in post-OPV era  
2. Inclusion of Sabin-IPV specifications |
| BCG (TRS 745, 1985)  
Hepatitis B (TRS 786, 1989)  
Acellular pertussis (TRS 878, 1998) | Recommended by ECBS  
Recommended by ECBS  
Recommended by WHO informal consultation (April 05) |
| Proposed new standards | |
| Meningococcal A conjugate | Clinical trial applications for countries in African meningitis belt expected before end 2007 |
| Stability evaluation of vaccines | Recommended by ECBS |
| Human papilloma vaccines | Anticipating strong interest from countries in vaccine introduction projects and need for prequalification |
| Combination vaccines | Increasing evidence of complex quality interactions in combination formulations coupled with continued trend for new combination applications to national regulatory authorities |
| Projects to be started in 2006–2007 (will become priorities for establishment by ECBS in 2008–2009) | |
| Live influenza vaccine (TRS 638, 1978) | New vaccine licensed in one country; not covered by the current document |
| New adjuvants | Broad range of new adjuvants in development |
| Inactivated Japanese encephalitis vaccine (TRS 771, 1988) | New cell culture vaccines in development |
1b. Guidelines to be established by the ECBS, or Working Groups active in 2006–2007 to improve safety of biologicals

<table>
<thead>
<tr>
<th>Document</th>
<th>Justification for assignment as priority</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proposed revised standards</td>
<td></td>
</tr>
<tr>
<td>Cell substrate guidelines (TRS 878, 1998)</td>
<td>Recommended by ECBS, Global Advisory Committee on Vaccine Safety</td>
</tr>
<tr>
<td>GMP for biologicals (TRS 822, 1992)</td>
<td>Recommended by ECBS</td>
</tr>
<tr>
<td>Proposed working group activities</td>
<td></td>
</tr>
<tr>
<td>Safety of gene therapies</td>
<td>Serious adverse events (leukaemias) documented</td>
</tr>
<tr>
<td>Safety of virus vectors</td>
<td>WHO Informal Consultation (2003) recommended topic be addressed in 2–3 years</td>
</tr>
<tr>
<td>Safety of biotherapeutic medicines</td>
<td>Serious adverse events (aplastic anaemia; unwanted immunogenicity) documented</td>
</tr>
</tbody>
</table>

1c. Global strategies to be developed in 2006–2007

<table>
<thead>
<tr>
<th>Activity</th>
<th>Justification for activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>To promote and monitor implementation of norms and standards</td>
<td></td>
</tr>
<tr>
<td>Develop tools to assess the impact of WHO quality standards</td>
<td>Monitoring implementation currently done in an ad hoc fashion</td>
</tr>
<tr>
<td>Develop tools to promote WHO quality standards</td>
<td>WHO manuals dealing with testing procedures for vaccine potency require producing or updating</td>
</tr>
<tr>
<td>To facilitate access to and appropriate use of international biological reference materials for vaccines</td>
<td></td>
</tr>
<tr>
<td>Develop training materials</td>
<td>Current guidance (TRS 932) does not extend to use of reference materials</td>
</tr>
<tr>
<td>To globally align priorities with the WHO collaborating centres for biological reference materials</td>
<td></td>
</tr>
<tr>
<td>Develop agreed policies and priorities</td>
<td>Recommended by ECBS</td>
</tr>
</tbody>
</table>

ECBS, WHO Expert Committee on Biological Standardization; TRS, Technical Report Series.

Maintenance of capacity for production of MRC-5 human diploid fibroblast cells

Human diploid fibroblasts (HDFs) are cell lines with a maximum lifespan of approximately 70 population doublings. These cells are used for production of a number of licensed human vaccines. A cell seed of one HDF cell line, MRC-5, was established at population doubling level (PDL) 7 before the emergence of bovine spongiform encephalopathy (BSE) and associated
transmissible spongiform encephalopathy (TSE) regulatory issues. In practice, MRC-5 cells are often used at around population doubling level (PDL) 35 although it has been demonstrated that they will grow to PDL 50 without loss of viability.

Despite this lifespan restriction, early calculations of vaccine production capacity indicated that the total cell production capacity from the ampoules of MRC-5 cells stored in the master bank would easily provide for their application as production cell substrates for viral vaccines into the distant future. However, in reality much of this capacity is lost when low passage cells are provided for new manufacturing processes and a considerable proportion of the total replicative capacity of the cells lines is not utilized.

Although there are a number of alternative cell substrates that could be investigated (eg new alternative HDF cells, HDF cells immortalized with telomerase and tumour cell lines) it would take a long time to evaluate, to address regulatory issues and to implement use of alternative cell substrates in new vaccine manufacturing processes. The Committee therefore considered a proposal from the National Institute for Biological Standards and Control (NIBSC) to anticipate the eventual depletion of MRC-5 early passage stocks by two means, firstly by establishing new seed stocks of the existing HDFs and secondly by revisiting the current constraints on maximum population doubling limits for production (WHO/BS/05.2012).

NIBSC proposes the establishment of a new seed stock of MRC-5 cells utilizing six ampoules at PDL 7 to generate 384 ampoules at PDL 12. This, on the basis of replies received from manufacturers, is considered sufficient to meet the current demands of manufacturers of viral vaccines and their

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Status</th>
<th>Target for end 2007</th>
<th>Target for end 2009</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of new or revised standards and reference materials established by the WHO Expert Committee on Biological Standardization</td>
<td>0</td>
<td>15</td>
<td>30</td>
</tr>
<tr>
<td>Number of new guidelines established, or globally coordinated research projects under way, that contributes to improved safety of biological medicines</td>
<td>0</td>
<td>5</td>
<td>8</td>
</tr>
<tr>
<td>Number of global strategies developed to promote and monitor implementation of norms and standards and to facilitate access to WHO collaborating centres.</td>
<td>0</td>
<td>3</td>
<td>6</td>
</tr>
</tbody>
</table>
anticipated needs in the near future. It is anticipated that the complete history of any serum used in this renewal process would be available.

NIBSC envisage that after consensus from manufacturers on this approach and the preparation of the seed stock in late 2005, the testing and qualification regime for the new stock of cells facilitated by WHO would be undertaken in 2006. A complete testing programme and test data would be presented to the committee in 2006.

The committee agreed with the proposal to prepare a new bank of MRC-5 cells under GMP at PDL 12 but advised NIBSC to seek scientific advice from key regional regulatory authorities and to investigate the regulatory implications of this proposal before proceeding.

**Blood products and related in vitro diagnostics**

The Committee was informed about the strategy of WHO for strengthening national regulatory authorities, building technical capacity to address specific risks and promoting the formation of regional networks of national regulatory authorities. The activities of WHO in 2004–2005 were summarized, and attention was drawn to the proposals for establishing Recommendations for the production, control and regulation of plasma for fractionation at this meeting, which included guidance on plasma from apheresis and whole blood donations. The document was also intended to provide guidance for the increasing number of Plasma Contract Fractionation Programmes and on the need for the implementation of good manufacturing practice (GMP) in blood establishments.

A high level WHO–European Commission (EC) meeting on IVDs was held on 21 April 2005 in Brussels to advocate the adoption of the WHO Biological Reference Materials for IVDs within the EC legal framework. As an example of coordination and harmonization of biological reference materials, the EC has agreed to adopt the second WHO International Standard (a global reference preparation) for hepatitis B surface antigen (HBsAg) in the category of higher order reference material. This action establishes the principle that WHO reference materials may be regarded as higher order reference materials. While it welcomed this development, the Committee nevertheless stressed that urgent action is needed to ensure that other WHO International Standards for the control of in vitro diagnostic procedures will be taken as higher order reference materials within the Common Technical Specifications of lists A and B supporting the European Directive on in vitro diagnostics.

A consultation meeting was held at WHO headquarters in September 2005 on tissue infectivity distribution in TSEs, and the risk of transmission of vCJD through blood and blood products. An update of the TSE tissue
infectivity categories, first published by WHO in 2003, was agreed in order to enable an assessment of the risk of transmission of vCJD via blood and blood products and the selection of measures to avoid the as yet unknown ways in which the disease might spread. The updated document provides evidence-based information to Member States, especially those countries where surveillance systems for BSE and vCJD are not yet in place.

The Committee received a report on a WHO Regional Workshop on Quality Assurance and Safety of Plasma and Plasma Derivatives held in Cairo, Egypt from 31 January–3 February 2005. In feedback from the course WHO was urged to promote consistency in the Region in respect of regulations, licensing and inspection of blood establishments and plasma fractionation facilities. The need for a network of national regulatory authorities within the Region in order to promote harmonization of regulatory policies was agreed upon. A Steering Committee was formed, comprising representatives from the national regulatory authorities of Bahrain, Egypt, the Islamic Republic of Iran and Tunisia that should facilitate developments in this context. The need for adherence to GMP throughout all steps from collection of starting material to manufacture and release of product to assure the safety, quality and efficacy of blood components and plasma derivatives was stressed. Member States should adopt and apply common regional guidelines on GMP standards for the provision of safe and effective blood, blood components and plasma for fractionation. Independent quality control of blood screening tests should be considered by national regulatory authorities. The appropriate selection of tests is a key intervention to assure safety of blood products.

The shortage of production of animal-derived sera (antisera) for therapeutic use was highlighted. Antisera are largely unavailable, consequently, high rates of mortality and morbidity are still a reality for some otherwise treatable conditions. Improvements are urgently needed in some production systems in developing countries and overall there is a need to provide appropriate regulatory controls.

The Committee was informed of a meeting held in May 2005 in Bethesda, MD, USA of key regulators to further develop the concept agreed at the last meeting of the Expert Committee on Biological Standardization for the establishment of the forum of regulators with regulatory oversight of blood, labile blood products and plasma products. The outcome of the meeting and the further development of this group are described in detail later in this report.

The Committee noted the priorities identified by WHO to strengthen the regulation and regulatory oversight of the quality and safety of blood products, haematological products and IVDs worldwide. However, the Committee strongly reiterated its opinion stated in 2004 that human and
financial resources available at WHO for work in this important field of global health remain inadequate and urgently need to be addressed.

**Advancement of technical expertise of regulatory authorities in the area of blood products and in vitro diagnostics**

The Committee was reminded of the recommendation made at its 2004 meeting, for WHO to promote worldwide cooperation of national regulatory authorities to facilitate improvement of their technical expertise regarding blood, blood products and IVDs, and to establish a global network of regulatory authorities. At this meeting, the Committee encouraged WHO to take appropriate steps for realization of the global network by developing, as a first step, the terms of reference. It also advised that the group, when formed, should report to the Expert Committee on Biological Standardization. The Committee was informed of the progress that had been made. A first meeting was held in Bethesda, MD, USA, in May 2005 to discuss the objectives of the group. These were agreed to be:

— scientific assessment of current and emerging threats to the safety and availability of blood products;
— scientific assessment of the impact, i.e. potential benefits and drawbacks, of new technologies in the field of blood products;
— exploration of opportunities among regulatory authorities to cooperatively address emerging public health challenges;
— exploration of opportunities for regulatory harmonization particularly in response to emerging public health challenges (eg actions to prevent transmission of vCJD, tools for removing TSE agents, and virus inactivation in blood components).

The Committee affirmed its decision of 2004 that WHO would establish a group for regulators, with the objectives outlined above, and agreed that it should be called the WHO Blood Regulators Network. The Committee also recommended that the network should report to it, and that WHO should provide some support for the activities of the network. The Committee further recommended that WHO should invite countries that have expressed an interest in joining the network and that the group would benefit from some representation from developing countries (eg chairs of regional blood regulators networks).

**Quality, safety and efficacy of animal plasma-derived antisera**

The Committee was informed of the important role of specific therapeutic treatments (eg snake antivenoms) and passive immunizations for some infectious diseases (eg rabies immunoglobulins and diphtheria antitoxins) that are based on antisera derived from the plasma of immunized animals. Although such products have been used for many years, there are a number
of current problems. There has been a sharp reduction in the number of manufacturers of animal antisera and some production systems in the developing world are fragile. In some places there is poor regulatory control over the manufacture of antivenoms and over the import of such products. Furthermore, access to antisera was in some cases hampered by high costs; logistical problems in distributing animal sera; and a general lack of knowledge about prevention and correct medical management of diseases that could effectively be treated by antisera.

A review of the potential risk of transmission of animal viruses to humans from the use of animal sera concluded that no cases have been attributed to these products. It is very likely that, by analogy to the manufacturing process for products from human plasma, that current manufacturing steps for animal sera have the potential for virus inactivation and/or virus removal, but may not be recognized as such by the manufacturers. Improvements to methods of manufacturing animal antisera can be identified that may reduce the clinical side-effects linked to animal proteins. The Committee cautioned though that the impact of any changes to a manufacturing process on product efficacy should be carefully considered.

The Committee was presented with a series of proposals for possible action by WHO, namely, to support production of antisera in the developing world according to GMP; to support viral validation studies of existing manufacturing steps when needed; to reinforce the capacity of local NRAs and to establish a pre-qualification system for antisera following the existing WHO experience in prequalification of other therapeutic products. The proposed activities should be targeted to the local NRA and local manufacturers, and they may be organized at the regional level to facilitate development of regional NRA networks. The Committee advised focusing on those activities that would have most impact, i.e., GMP for antisera, good animal husbandry practices, collection practices for animal plasma, and implementation of existing manufacturing steps possibly contributing to viral safety (eg caprylic acid precipitation, low pH treatment).

The Committee was also presented with a proposal to develop guidance and training materials on the prevention, diagnosis and management of diseases treatable by antisera (especially envenomations) and that WHO recommendations on the production, control and regulation of animal plasma-derived antisera should be developed. These could be elaborated in parallel with, and as a result of, regional workshops which would also contribute to the goal of education of local inspectors. These activities will enhance the visibility and awareness of the therapeutic importance of antisera and support the production and availability of safe antisera products.

The Committee agreed with the planned activities and urged WHO to mobilize resources to support this area of work.
Global needs in standardization of products derived by biotechnology

The Committee was informed that control of chronic diseases was an increasingly important global public health problem that needed to be acted upon urgently. The means for preventing and controlling most chronic diseases are well established, and include biotechnological interventions. The number of approved innovative biotherapeutic products is expected to increase substantially over the coming years (e.g., some 500 monoclonal antibody-based products are currently in the pipeline in the European Union (EU) and USA alone). In addition, the imminent expiration of the patents on many biotechnology products will result in a substantial increase in “follow-on” or “biosimilar” products.

WHO is receiving requests from countries for advice on appropriate regulatory oversight for biological therapeutic products, because the potential for the success of therapeutic biological products used in the treatment of a wide variety of chronic diseases is being tempered by concerns over the quality, safety and availability of such products, leading to uncertainty for national regulatory authorities. The Committee heard that inappropriate assay methods or poor potency determinations (e.g., for recombinant streptokinase) can lead to life or death clinical problems; and adverse drug reactions, for example unwanted antibody development in some individuals, could occur following administration of a variety of products. Increased risks of infections have been seen with blockers of tumour necrosis factor \( \alpha \) and the potential for substandard and counterfeit biotech products is an important cause for concern.

The Committee received a report on the existing WHO written standards and reference materials that are, or may be, relevant to biological therapeutic products, and on the support for the science base in this area provided through activities such as the series of WHO informal consultations on standards for cytokines, growth factors and endocrinological substances and a WHO working group on gene therapies.

The need for further WHO initiatives was discussed. These included updating of existing WHO written standards, the establishment of new WHO written standards to promote and develop appropriate understanding of the risks and benefits of biotechnological products, continued development of new reference materials and strengthening of technical capacity in national regulatory authorities in this area.

The Committee took note of the proposed activities and agreed that WHO should organize a meeting of interested parties to review the issues in depth and help WHO develop consensus on the global needs, priorities and potential role for global standardization in the area of biotherapeutics.
for the major chronic diseases. The Committee also recommended that WHO should facilitate the strengthening of technical capacity of national regulatory authorities for regulating biological therapeutic products and also collate information on substandard or counterfeit biological medicines for the treatment of chronic diseases.

**International Non-proprietary Names for gene therapy products**

An International Nonproprietary Name (INN) is a unique global name for a single pharmaceutical substance. The use if INNs was initiated in 1953 and they are perceived as an essential component in the regulatory process of many countries (eg the EU, Japan and the USA). An INN is required for licensing of products and although assigned to certain biologics, particularly biotechnology products (such as rDNA products and monoclonal antibodies), by convention they are not assigned to vaccines or natural blood products.

In January 2005, the WHO Executive Board emphasized the importance of INNs for global public health programmes. However the Board decided that INNs should only be considered for products at the phase III of clinical development and only for products already in commercial development.

The Committee was informed that WHO had held a consultation in January 2005 on INNs for gene therapy products. The participants had agreed that there was a need for INNs for gene therapy products and had proposed a possible systematic nomenclature scheme for such products. However the experts advised that the proposed system should not apply, at this stage, to other gene transfer products such as DNA vaccines or vaccines based on live viral vectors.

The scope of gene therapy products is very wide and many trials are being undertaken globally. These involve a wide range of types of transferred genes such as antigen, tumour suppressor, cytokine, drug resistance, deficiency correction and others. A range of different types of gene therapy vectors are also currently used e.g. adenoviruses, adeno-associated viruses, herpes simplex virus, certain poxviruses, retroviruses, lentiviruses and plasmid DNA.

It was agreed that gene therapy has become a clinical reality and that the INN nomenclature system would contribute to patient safety, provided that both the vector and the gene transferred could be rapidly identified. Both United States Adopted Names (USAN)/US Food and Drug Administration (FDA) and the European Agency for the Evaluation of Medical Products (EMEA) had given much thought to possible nomenclature systems, and it was agreed to build on the experience obtained with other complex biologics (such
as fusion proteins). Therefore the consultation recommended that an INN system for gene therapy products should be based on a two word scheme. The first word would define the expression gene and the second the vector component.

The first word to describe the expression gene would have a prefix which would be a distinctive name e.g. al-, bel- or val-; an infix which would identify the gene using existing infixes as for the protein for which the gene codes; and a suffix — gen or gene. The second word for the vector component would have a prefix which again contributes to a distinctive name; an infix e.g. lenti (lentivirus) or retro (other retroviruses) or adeno (adenovirus); and a suffix to indicate the viral vector “vec”. The consultation agreed that for medicinal products administered by transfecting a patient’s cells ex vivo, the cells themselves were considered as the route of administration only, and not included in the INN.

The Committee noted this proposal and that further work may be required to refine the recommended INN policy as experience is gained with applications for gene therapy products.

Reports from the WHO International Laboratories and WHO Collaborating Centres

The Committee was informed of recent developments at the various WHO International Laboratories and Collaborating Centres for biological standardization.

**National Institute for Biological Standards and Control, Potters Bar, England**

The Committee was provided with an updated overview from the National Institute for Biological Standards and Control (NIBSC) regarding the contribution of the Institute to the WHO biological standardization programme. The Director, Dr Inglis, outlined significant changes to the way in which NIBSC would be managed by the UK Department of Health. He stressed the need for, and was assured of, strong support from the Committee to ensure that under the new management structure the NIBSC “brand” was protected and also that maintaining a full spectrum of activities across all biological medicines, and not just work on vaccines, was of critical importance for global public health reasons.

The Director gave an overview of the standards programme currently in progress. In 2004–2005 approximately 5000 shipments of biological reference preparations were made, representing increase on previous years of about 10%. The overall inventory of such standards is now over 2 million vials and ampoules. Ongoing work includes 83 active standards development projects, 61 projects already endorsed by the Committee
and 22 new projects identified since 2004. A complete list of all projects designed to capture all the relevant information and providing a resource for retaining knowledge over time is now established and was provided to the Committee. The database provides a tool for the management of projects through regular reporting of progress, ensuring that key steps are followed, and resource planning for example in statistics and filling suite capacity. The list will be reviewed annually by project leaders together with the WHO secretariat in preparation for the meeting of the Committee. New projects will be brought to the Committee for discussion and endorsement. The 22 new projects identified since 2004 were agreed by the Committee.

The alignment between the NIBSC programme and potential priorities for new or replacement reference preparations identified by WHO was reviewed. In most cases there was a good match, but there were some exceptions. The Committee therefore recommended further discussion on the need for WHO international standards for rotavirus, meningococcal A antisera; meningococcal B antisera; PCR-enhanced reverse transcriptase (PERT) testing, and a standard for residual cellular DNA levels in biological medicines.

The Committee received a report on progress made at NIBSC in developing training in biological standardization. A training course was currently being piloted. The intention was to construct a lecture based course in the principles of biological standardization to be supplemented by subsequent practical training through placements. In the long term a manual on biological standardization was envisaged.

The risks to global biological standardization if NIBSC were to suffer a catastrophic loss of stocks were also discussed. Offsite storage of a limited number of important reference materials has already been undertaken.

The Committee was informed about the ongoing and projected work on standards in haemostasis and thrombosis from 2005 to 2009 at NIBSC. Highest priority is given to replacement standards. For new standards, highest priority is generally given to new therapeutic materials that are, or are about to be licensed, and to important diagnostic tests.

Finally it was noted, and agreed by the Committee, that the implications of ISO 34 for the ECBS standards setting process need to be considered by WHO and the Collaborating Laboratories.

Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Sanquin), Amsterdam, the Netherlands

The Committee heard a presentation on behalf of the WHO International Laboratory at Sanquin Diagnostic Services. It reported that Sanquin had decided recently to discontinue custodianship for the WHO Biological
Standardization Programme by the end of 2006. The Committee was informed that the organization had transferred their own activities on biological standards to another organization. Infrastructure for the warehousing and shipment of biological standards will be reduced in the near future. Furthermore employees with expertise in biological standardization had left the organization and, strategically, Sanquin Diagnostic Services had decided to focus on their core activities, namely diagnostic services for the blood transfusion service and national health care needs. Various attempts had failed to convince the Dutch Government to continue funding of the important custodianship activity. The Committee recommended that WHO express appreciation to Sanquin for their work over the years as an International Laboratory, but also regret that they have decided to stop performing this function. Furthermore WHO should urgently review the broader implications for its biological standards programme of the loss of this laboratory as a collaborating centre.

Transfer of custodianship of the WHO reference preparations currently held by Sanquin to another party selected by WHO is required. Although Sanquin Diagnostic Services will cooperate with WHO in the proper transfer of the activities to this party, the transfer should be completed by the end of 2006 before the shutdown of the facilities.

The status of ongoing standards projects that are the currently the responsibility of Sanquin was reviewed. Sanquin gave an undertaking to complete a study of mono-specific anti-HCV antibodies. Sanquin has also started the preparation of the replacement standard for W1042 anti-hepatitis B immunoglobulin and will prepare a candidate replacement standard. The laboratory will also design a protocol for evaluation of the candidate standard together with another collaborating centre. However Sanquin requested that completion of the study be taken over by the “new” custodian.

The Committee noted that sustainable government funding of biological standardization activities had became increasingly difficult to secure in recent years, a development which may have significant impact for the biological standardization programme of WHO. A comprehensive advocacy programme to explain the benefits of biological standardization to policymakers was needed.

*Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD, United States*

The Committee was informed of CBER’s mission as a regulatory authority, its activities as WHO Collaborative Centre and of recent organizational changes including the transfer of certain products to the Center for Drugs Evaluation and Research (CDER). The latter included therapeutic biologicals, antibiotics, insulin and human growth hormone. It was also
noted that medical devices, with the exception of those used in blood banks, were the responsibility of the Center for Devices and Radiological Health.

CBER’s activities include collaborative studies for reference materials, research and testing for improving standardization and control of biological products, training of laboratory personnel, providing expertise to many WHO working groups and informal consultations, serving on evaluation teams assessing NRAs according to WHO criteria, and improving communications about biological medicines. CBER also acted as adviser on quality control and laboratory related regulatory issues to a PAHO network of quality control laboratories and national regulatory authorities.

The presentation highlighted the development by CBER of a West Nile virus panel for the evaluation of nucleic acid amplification tests, and an RNA subgroup panel for HIV-1 for use by manufacturers to assess compliance with sensitivity and specificity requirements. CBER had hosted scientists from Latin America for training in testing diphtheria and pertussis vaccines. The Committee recommended WHO to request CBER to consider the applicability of extending the training it offers to PAHO scientists in vaccine quality control to scientists from other parts of the world.

The current priorities for blood products included updated product specifications for leukocyte depleted platelets and HBsAg test sensitivity.

**WHO Collaborating Center for Research and Reference Services for Immunological and Biological Products, Japan**

The Committee was informed of the activities and different functions of the WHO Collaborating Center for Research and Reference Services for Immunological and Biological Products, Japan (NIID). As a WHO collaborative centre it focuses on four major items:

- standardization and recommendations on the calibration of test methods for the quality control of biological products and other related activities (e.g. laboratory tests replacing animals models);
- standardization and calibration of reference preparations for the quality control of biological products;
- training in methods for the quality control of biological products; and
- collaboration and consultation with other national and international standard-setting organizations.

Some of the recent activities of the NIID were outlined for consideration by the Committee. For example, attention was drawn to the fact that various viruses and certain vaccines, such as whole virus influenza vaccine, may be associated with leukopenia. A mouse model that is used in Japan to assess the leukopenic activity of vaccines and monitor consistency of production was described. This is an example of research efforts towards developing
clinically relevant laboratory test models which, together with postmarketing surveillance of adverse effects, may contribute towards the improvement of quality assurance systems for biological products. The NIID representative proposed that the Expert Committee on Biological Standardization, as well as each NRA, should make concrete plans on a budget for promoting quality control activities for biological products.

The NIID representative expressed support for the WHO/Expert Committee on Biological Standardization initiative to develop Regional Working Reference Standards in each Region. It was suggested that this might contribute to reducing the heavy workload of NIBSC involved in providing numerous International Reference Standards. He presented an example of an existing regional cooperation between China, Japan and the Republic of Korea, in quality control of biological products and infection control and surveillance leading to the establishment of a regional reference standard for mamushi (*Gloydius blomhoffii*) antivenom. The Committee recommended that WHO request NIID to provide further details on the methods used to standardize this reference material, to see if the principles used might be generally applicable.

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

The Committee was informed of the approval of the institute as a WHO Collaborative Centre on 6 June 2005 by WHO. A report was received on the activities of the Paul Ehrlich Institute in quality assurance of blood products and IVDs and the contribution of the Institute in the development of WHO’s strategy for safe blood/plasma-derived medicinal products through GMP and educational tools for blood collection establishments. The Institute trains inspectors in GMP at the regional level, and participates in workshops on aspects of blood transfusion, licensing and batch release of biological medicinal products.

The Paul Ehrlich Institute offers training courses for assessors working in national regulatory authorities on the quality assurance of blood products and IVDs. For blood products, training topics include bacterial and viral safety, pyrogen testing, immunoglobulins, antisera, monoclonal antibodies, coagulation factors, albumin and other blood products. For IVDs, training topics include control of serological tests, control of nucleic acid amplification tests and regulatory oversight.

The Paul Ehrlich Institute contributes to the development of guidelines and recommendations and to the quality assurance of IVDs. It also contributes to the human and financial resources available to WHO through, for example, secondment of a staff member to WHO, waiving fees for courses, or covering its own expenses for the majority of WHO activities.
Finally it was noted that various biological reference materials had been developed at the Paul Ehrlich Institute. The Committee recommended that WHO should review the suitability of these reference materials as potential WHO International Standards.

Taking into account all of the above reports from collaborating centres, the Committee recommended that WHO should:

1. collectively negotiate with the collaborating centres to ensure there is agreement on which collaborating centre will take forward which new reference material project, and
2. collate and review the training activities under way in each collaborating centre to ensure maximum efficiency and synergy in training activities.

**Feedback from users of WHO biological standardization products**

The Committee was informed of the activities by the WHO South-East Asia Region in the area of biological standardization through a project to develop Regional Working Reference Standards for vaccines. This was being developed by a Regional National Control Laboratory Network involving the three vaccine producing countries of the Region (India, Indonesia and Thailand) and was discussed at a WHO meeting held in Bangkok, Thailand in November 2004. Based on regional public health needs and regional priorities, the laboratories agreed to initiate work to develop Regional Working Reference Standards for whole cell pertussis vaccine and inactivated Japanese encephalitis vaccine.

The meeting in Bangkok had recommended that WHO should develop advocacy material for biological standardization and also conduct an assessment of the feasibility of establishing regional biological standards in South-East Asia. The Committee was informed of the outcome of the feasibility assessment. It was concluded that the initiative was indeed feasible and would contribute significantly to strengthening regional collaboration in the regulation of vaccines. A recommendation was made that consideration should be given to expanding the planned activities to include additional countries. Parallels were drawn with an already established regional chemical standards programme that included not only countries of the WHO South-East Asia Region, but also countries from the adjacent WHO Western Pacific Region. Establishing cross-regional collaborations for biological standards would, in time, become very beneficial. Moreover during the course of the feasibility assessment it had become apparent that an inventory of critical reagents for batch release activities for vaccines would be useful, and WHO should explore the possibility of helping to source these materials. It was concluded that the priorities for use of resources should be training activities rather than laboratory facilities such as filling capacities for standards.
A need for increased geographical representation in WHO collaborative studies from laboratories was raised in discussion. Consequently the Committee requested WHO to consider publishing the details of forthcoming collaborative studies and requesting expressions of interest in participating in the studies. The Committee also acknowledged the important role that proficiency studies may play in increasing capacity and so recommended that WHO should investigate the inclusion of proficiency testing for vaccines within the South-East Asia Region national control laboratory network.

**International guidelines, recommendations and other matters related to the manufacture and quality control of biologicals**

**Guidelines for assuring the quality and nonclinical safety evaluation of DNA vaccines**

A new approach to vaccination involving the direct introduction of plasmid DNA containing the gene encoding the antigen against which an immune response is sought into appropriate host tissues with the in situ production of the target antigen(s) has been developed in the past 10 years. This approach offers a combination of potential advantages over the more traditional approaches, including the stimulation of both B and T cell responses, improved thermal stability of the vaccine, absence of adventitious agents and the relative ease of large scale manufacture. Many scientific publications have addressed the potential of DNA vaccination and immune responses in animal models have been obtained using genes from a variety of infectious agents including influenza virus, hepatitis B virus, human immunodeficiency virus, rabies virus, lymphocytic choriomeningitis virus, malaria and mycoplasma. However, the immune responses observed in animal models have generally not been reproduced in humans when phase I clinical trials were undertaken and many approaches have been and are being followed in order to enhance the human immune response.

DNA vaccines have been licensed for veterinary use and efficacy in animal target species has been observed. Although the quality and safety considerations for vaccines for veterinary use differ from those for vaccines for human use, experience with veterinary DNA vaccines can provide valuable information for the control and use of human DNA vaccines.

A revision of the WHO guidelines for DNA vaccines was initiated in 2002 to reflect the rapidly accumulating knowledge on these vaccines and an updated draft was considered in detail in July 2005 at a WHO Consultation attended by regulators, industry representatives and academic experts. On the basis of this meeting, a proposed revised version of the guidelines had been
prepared (WHO/BS/05.2013) and was considered by the Committee. This revision includes information on plasmid DNA vaccines for prophylactic and therapeutic use but does not cover plasmid DNA vaccines for use in gene therapy, DNA vaccines derived in eukaryotic cells, bacterial vectored plasmid DNA vaccines or synthetic oligonucleotides.

These guidelines are intended to provide information and guidance to national regulatory authorities and vaccine manufacturers concerning the characteristics, production, quality control and nonclinical development of DNA vaccines. The section on nonclinical development had been drawn up in response to the use of different experimental approaches to enhancing the efficacy of DNA vaccines, which may raise specific safety concerns.

After making suitable amendments, the Committee recommended adoption of the guidelines and agreed that the text should be appended to its report (Annex 1).

**Recommendations for rabies vaccines**

The last revision of the WHO requirements for rabies vaccines for human use was in 1980 although an additional document, WHO Requirements for rabies vaccine (inactivated) for human use produced in continuous cell lines was published in 1987 to take into consideration advances in the development of cell culture derived vaccines. An amendment which updated the section on the International Standard for Rabies Vaccine was published in 1994. Since these two sets of requirements for rabies vaccines were published there have been many developments in the production and quality control of vaccines as well as in their overall regulation. In particular, safety issues have been carefully reconsidered over the last 10 years.

The availability and use rabies vaccines produced in cell culture or purified from embryonated duck egg has dramatically decreased the number of human deaths from rabies worldwide, but most notably in countries where canine rabies is endemic. The revised scope of the WHO recommendations (WHO/BS/05.2015) encompasses vaccines produced in cell substrates, ranging from primary cells (hamster kidney and chick embryo fibroblasts), human and monkey diploid cells (MRC-5 and FRhL-2 cells), to continuous cell lines such as Vero cells. Vaccines purified from duck embryos are also within the scope of the document. However, neural tissues are no longer considered as suitable substrates for the production of rabies vaccine.

Relevant guidance documents published since the last revision of the requirement for rabies vaccines have also been considered in this revision, including the WHO guidelines on animal cell substrates which include the revised requirement for not more than 10 ng of residual host cell DNA per single human dose.
In addition to the substrates for vaccine production, the major issues addressed in this revision are biosafety levels used for production and quality control areas; testing for adventitious agents; the genetic characterization of virus strains used in vaccine production; the inactivation process; the test for effective inactivation; the use of in vitro assays for determination of the antigen content as a measure of consistency of production; and potency tests. The stability of the final product and intermediates, guidance on the quality control of vaccines to be administered by the intradermal route and clinical evaluation of vaccines are also addressed in separate sections.

The Committee, after making suitable amendments, advised that the recommendations are adopted and appended to its report (Annex 2). In addition, in view of the extensive text on testing for adventitious agents in this document, the Committee recommended that the Secretariat consider development of a separate document on testing for adventitious agents. This would facilitate consistency between all WHO guidance documents which include testing for adventitious agents.

**Guidelines to assure the quality, safety and efficacy of live attenuated rotavirus vaccines (oral)**

The high incidence of rotavirus disease around the world has prompted international agencies and partnerships including the WHO, the Global Alliance for Vaccines and Immunization (GAVI), and the Children’s Vaccine Program at the Program for Appropriate Technology in Health (PATH) to identify rotavirus vaccine development as one of their highest priorities. Examples of vaccines approved or in development include multivalent human–bovine and human–rhesus reassortant vaccines and monovalent vaccines containing a single attenuated human rotavirus strain, natural bovine–human reassortants and animal rotavirus strains.

A WHO Consultative group met in February 2005 to review the scientific basis for quality, safety and efficacy of live attenuated rotavirus vaccines. Issues considered included the control of vaccine source materials and vaccine production, nonclinical and clinical evaluation of vaccines based on existing WHO guidelines in general and issues of quality, safety and efficacy specific to rotavirus. Draft guidelines (WHO/BS/05.2014) were subsequently developed and reviewed at an WHO informal consultation in August 2005.

Issues relevant to the production and control of rotavirus vaccines are the sensitivity of the virus at acid pH; testing for adventitious agents and qualification of the vaccine source materials; the need for a full passage history of the seed materials to identify all substrates through which they have been passed; the levels of impurities such as residual cellular DNA; assays to assess the concentration of virus throughout the production process; and the applicability of suitable reference materials which are likely
to be product-specific; and stability of the final product in the presence or absence of the vaccine diluent which is added prior to vaccination.

Claims of attenuation of the strains used in the production of rotavirus vaccines are based on clinical experience in human subjects rather than on animal models which are not readily available or laboratory markers of attenuation which are not well-defined. Potential laboratory markers of product consistency include genetic sequence information which may demonstrate that a new virus seed is similar to the previous seed and that each could be distinguished from the parent virus.

Clinical issues are considered in detail in the draft guidelines and, although many of the points of possible concern are generic, the guidelines emphasize that each candidate needs to be examined individually as there may be significant product specific issues. These will include the dose required to result in immunization, the possibility of transmission to contacts of vaccinees and the genetic stability of the virus on replication in the gut of recipients.

The Committee agreed that the use of primary cells for the production of rotavirus vaccine should be discouraged, as primary cell cultures prepared from wild animals often show a high frequency of viral contamination.

After making suitable amendments, the Committee agreed that the Guidelines should be adopted and appended to its report (Annex 3).

**WHO Biosafety guidelines for the production and quality control of human influenza pandemic vaccine strains**

WHO guidance on the production of pilot lots of inactivated influenza vaccines from reassortants derived from avian influenza viruses was prepared in 2003 in response to the threat of a pandemic posed by highly pathogenic H5N1 avian influenza viruses and the need to begin development of experimental vaccines. This threat still persists and several countries are planning large-scale production of H5N1 vaccine. The earlier guidance has therefore been reassessed in light of the larger scale of intended production and also in light of the experience gained from developing and testing vaccine reference viruses derived by reverse genetics from highly pathogenic avian influenza viruses.

Revised guidelines had thus been prepared (WHO/BS/05.2026) which detailed international biosafety expectations for large-scale vaccine production and control and specified steps to minimize the risk of introducing influenza virus strains with pandemic potential from a vaccine manufacturing facility into the community. The risk assessment approach used previously for production of pilot lots of vaccine was followed. However, it is anticipated that the risks associated with large scale production are likely to be different from those associated with pilot lots, e.g. the “open” aspect of some production processes and the quantity of virus-containing waste. Moreover the revised document
had been expanded to encompass current vaccine development pathways. The guidelines consider a range of hazards associated with manufacturing and laboratory testing of vaccines for use during a pandemic which are dependent on the type of virus strain used for production (reassortant versus wild type), method of production (egg-based versus cell-based) and intended viability of the virus in the product (inactivated versus live attenuated).

Although the guidance was influenced considerably by the experience gained with the H5 strains, it will also be applicable to future threats of pandemics from other potential pandemic strains, such as H2 or highly pathogenic H7. In addition it covers the possibility that live attenuated virus vaccines, as well as inactivated vaccines, may be developed as potential pandemic vaccines.

The Committee, after making suitable amendments, advised that the recommendations should be adopted and appended to its report (Annex 5). The Committee also recommended that some of the matters raised in the document be discussed in an international regulatory meeting.

**Recommendations for whole cell pertussis vaccines**

The last revision of requirements for diphtheria, tetanus, pertussis (DTP) and combined DTP vaccines was in 1989. Since that time, there have been a number of developments in the methods of production and quality control of whole cell pertussis vaccines. Progress was reviewed by a WHO working group on several occasions (Bethesda, USA, November 2000; Geneva, Switzerland, July 2003) and key issues presented to the Committee in 2004. A consensus on a scientific basis for the revision of the requirements was reached at the Informal Consultation on the Standardization and Control of Pertussis Vaccines, held in Geneva in March 2005. The main changes that were incorporated into a draft revised standard (WHO/BS/05.2016) include an update on reference reagents; the use of the mouse weight gain test to assess specific toxicity; a clarification of the estimated potency; and new sections on stability, nonclinical and clinical evaluation of whole cell pertussis vaccines. In addition, a number of clarifications on technical details of various tests as well as on the interpretation of the results were provided.

This revision of the recommendations for whole cell pertussis vaccine is part of the revision of recommendations for DTP and should be read in conjunction with the revisions already made for diphtheria and tetanus vaccines (WHO Technical Report Series, No. 927, 2005, Annex 5).

The Committee, after making suitable amendments, advised that the recommendations should be adopted and appended its report (Annex 6).

In addition, the Committee encouraged surveillance of pertussis strains to further evaluate the effect of vaccination on circulation of wild-type strains, and carriage of strains in populations.
Recommendations for the production, control and regulation of human plasma for fractionation

Human plasma is a source of important medicinal products which are obtained by a combination of large-scale processing steps called “fractionation”. It is important that these products have an optimal quality and safety profile. The current WHO requirements for the collection, processing, and quality control of blood, blood components, and plasma derivatives were published in 1992 (WHO Technical Report Series, No. 840, 1994). Subsequently, supplementary guidance was developed on specific topics, such as the recently published WHO guidelines on viral inactivation and removal procedures (WHO Technical Report Series, No. 924, 2004), to address the measures necessary to eliminate blood-borne viruses during processing of plasma into plasma derivatives.

A drafting group reviewed and revised those parts of the 1992 Requirements dealing with plasma for fractionation, and prepared draft Recommendations for consultation (WHO/BS/05.2019). The intended purpose of the new document was to provide guidance, to both developed and developing countries, on the production, control and regulation of human plasma for fractionation as a source material for plasma derived medicinal products.

The Committee considered that the document, by summarizing experience and information, would serve as a guide to blood establishments to enable them to understand and facilitate the implementation of appropriate procedures for the production and control of the starting plasma material, and to facilitate the provision of safe fractionated plasma products at country level. It should also be helpful in establishing the supervision by the national regulatory authorities in their role of assessing the quality and safety of plasma, prepared either locally or imported, for fractionation, therefore contributing to the improved quality and safety of human plasma products worldwide. Manufacturers of plasma derivatives would also benefit from the document which would provide a common worldwide expectation for the quality criteria to be applied to plasma for fractionation.

The Committee discussed the proposed Recommendations in detail and, after making suitable amendments, advised that the Recommendations should be adopted and appended to its report (Annex 4).

WHO guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products

The Committee was informed of the work being done to revise the existing WHO guidelines on transmissible TSEs to reflect new scientific knowledge. A variant form of Creutzfeldt–Jakob disease (vCJD), a fatal brain disease of humans, first appeared in the mid-1990s, as a result of the epidemic
of bovine spongiform encephalopathy (BSE or “mad-cow” disease) in the United Kingdom. Since first reports in 1996, 157 cases of vCJD have been reported in the United Kingdom, 14 in France, three in Ireland, and single cases in Canada, Italy, Japan, the Netherlands, Portugal, Saudi Arabia, Spain and the USA. Cases of BSE and vCJD have been decreasing in the United Kingdom in recent years, but both diseases have appeared in other countries.

Until recently, all vCJD cases were attributed to consumption of beef products contaminated with the infectious agent of BSE. Since December 2003, two individuals have been identified with vCJD infections probably acquired from blood transfusions—one with typical vCJD symptoms and the other with preclinical vCJD involving the spleen and lymph nodes but not the brain. The fact that the two vCJD infections followed transfusions of blood from clinically healthy persons who became ill more than a year after donating blood implies that other blood donors currently incubating the disease might also be potential sources of infection for recipients. The possible extent of future blood-borne spread of vCJD infections is still unknown. The identification of these cases has intensified the concern about previously unconsidered ways in which the disease might spread. Except for the two transfusion-transmitted infections, no cases of vCJD have been linked to any medicinal product to date, and guidelines from WHO and other authorities have been in place for many years to minimize risk from such products.

A meeting held at WHO, Geneva, in September 2005 brought together experts and regulators from around the world to revise the existing WHO Guidelines on Transmissible Spongiform Encephalopathies (TSEs) in relation to Biological and Pharmaceutical Products, which provide guidance to both regulators and manufacturers on ways to prevent potential transmission of vCJD through human blood and blood products, as well as through medicinal products prepared with bovine derived materials. The primary aim of the meeting was to provide updated evidence-based information to the national regulatory authorities of WHO Member States, especially to those where BSE has not yet been reported and where surveillance systems for BSE and vCJD may not be in place. The information is intended to assist authorities in conducting risk assessments and selecting measures to reduce the risk of transmitting vCJD by human blood and blood products and other medicinal products of biological origin (biologics).

In 2003, WHO Guidelines encouraged authorities to consider introduction of precautionary measures to keep human blood and blood products safe from the then-theoretical risk of transmitting vCJD while continuing to ensure an adequate supply of these important medicines. The experts at the September 2005 meeting acknowledged that transfusion-transmitted vCJD
is no longer just a theoretical possibility and again advised authorities to assess the vCJD risk in the context of their own national situations, weighing the potential benefits of adopting precautionary policies to reduce that risk against the impact that policies might have on the supply of blood.

Earlier WHO meetings had repeatedly stressed that, when feasible, the use of tissues or body fluids of ruminant origin in the preparation of biologicals should be avoided. When bovine materials must be used, they should be obtained from sources assessed to have negligible risk from the infectious agent of BSE. Most bovine tissues, including bovine muscle, used to manufacture biologicals, if carefully selected by taking into account the geographical distribution of BSE and collected according to guidelines, have little risk of contamination with BSE agent. The recent findings of disease-associated proteins in the muscles of sheep with scrapie (a disease similar to BSE but not known to infect humans) and the recognition of BSE itself in a goat, reinforce the need for manufacturers of biologicals to maintain the precautionary safety measures laid down in the previous WHO guidelines. Ruminant blood and blood derivatives, such as fetal calf serum in cell cultures and bovine serum albumin stabilizers, are also used to prepare biologicals. Bovine blood has not been identified as a source of infection, and if properly collected, fetal bovine serum has a negligible risk. The blood of sheep with experimental BSE or natural scrapie can be infectious and, because scrapie and BSE agents behave similarly in sheep and goats, the use of the blood of small ruminants in preparing biologicals should either be avoided or animals selected very carefully from sources known to be free of TSEs.

There is a continuing need to ensure that all national regulatory authorities with limited resources have ready access to reliable and up-to-date information when assessing TSE risks and evaluating product safety. That information includes guidance to help minimize or eliminate the risk for transmitting BSE and vCJD to humans via biologicals and other medicinal products. The Committee recommended that the revised 2005 guidelines on minimizing TSE risk should be adopted as the current WHO position and be published on the WHO web site (www.who.int/biologicals).

Guidelines to assure the quality, safety and efficacy of human cells and tissues for transplantation

The Committee was given an introduction to the World Health Assembly Resolution 57.18 regarding allogeneic transplantation accepted in May 2005. The aims of the resolution are:

— to implement effective national oversight of procurement, processing and transplantation of human cells tissues and organs;
— to cooperate in establishing guidelines to harmonize global practices;
— to consider setting up ethics commissions to ensure the ethical use of cell, tissue and organ transplantation;
— to extend the use of living kidney donations when possible, in addition to donations from deceased donors;
— to take measures to protect the poorest and most vulnerable groups from “transplant tourism” and from the sale of tissues and organs, including giving attention to the wider problem of international trafficking in human tissues and organs.

The Committee was reminded that, during its previous meeting it had agreed with a proposal to develop global guidelines to assure the quality and safety of cells and tissues used in transplantation. The Committee was provided with an outline of the principles used to draft the guideline (WHO/BS/05.2010) namely to:

— build on existing international guidance;
— to distil out key fundamental requirements;
— to focus on tissue or cell products that are commonly traded across international frontiers; and
— to produce clear, user-friendly documents.

Accordingly, existing international guidance and regulations were reviewed to identify cross-cutting core requirements and to identify core requirements for specific tissues and cells. The resulting draft document aims to assist regulators, tissue bank operators and tissue and cell transplant surgeons globally by providing guidance on safety and quality requirements for the most used and distributed human tissues and cells. The specifications are product-based. They are designed to define international expectations for essential testing, donor selection and processing requirements for selected tissues or cells. They also provide guidance on the information to be provided with the product either on the label or in the accompanying documentation. The draft document did not include specific quality criteria because it was intended that these would be added in the form of an aide-memoire in the near future.

A list of tissue and cell products that are transplanted in the human body and that are used widely, and commonly cross international boundaries, has been established. This list was agreed at a global consultation with tissue and cell banking experts and regulators held in Ottawa from 29 November to 2 December 2004 and selection criteria and specifications were defined for the following products: frozen bone and tendon; freeze-dried bone; fresh, glycerolized or cryopreserved skin; fresh or glycerolized amniotic membrane; cryopreserved cardiac valve; fresh or culture-medium preserved cornea; fresh haematopoietic stem cells; and cryopreserved cord blood stem cells.
The Committee was informed that the draft guidelines represents a first step towards providing guidance from WHO in the field of human cells and tissue transplantation, and that the document will evolve with the expected rapid developments in the field. The Committee was concerned that no description of quality criteria was included in the document and that GMP aspects also needed to be addressed. Accordingly, the Committee recommended that the document should not be appended to the report of the Committee, but that the current draft (WHO/BS/05.2010) would be made available through other channels in the form of an aide memoire. The Committee also recommended that the current draft be further developed to include detailed guidance on quality requirements for cells and tissues and the role of national regulatory authorities in the inspection of facilities, and that a revised document should be resubmitted to the Committee.

**Guidelines for good manufacturing practice for biological products**

The Committee was reminded that GMP is that part of quality assurance which ensures that products are consistently produced and controlled to the quality standards appropriate to their intended use and as required by the marketing authorization. The principles of GMP apply to both drugs and biologicals. The main principles that apply to both classes of product are set out in WHO Good Manufacturing Practices (WHO Technical Report Series) and were last revised in 2003. These technical standards are used in many Member States for product and establishment licensure and are also used as the basis for assessing compliance for prequalification of vaccines for supply to United Nations agencies.

The WHO GMP includes an annex specifically for biological medicines which was last updated in 1992. Taking into account the developments in the field since then, the Committee was asked to decide if there is a continued need for specific WHO GMP guidance for biological products and if so, if all areas of biological medicinal products are adequately covered. Topics that might be addressed included blood and plasma-derived products; production of microorganisms requiring biological containment; gene and tissue therapies; biological products containing biotechnology-derived proteins (GMOs); computerized systems (which would not be unique to biologicals but would need to be developed in collaboration with colleagues responsible for GMP of chemical medicines); animal testing for batch release purposes; and a classification system for GMP deficiencies for biological medicines.

The Committee expressed its firm opinion that the application of GMP in the production of all pharmaceuticals including biologicals is critical and that the regulations need to be kept up to date. The Committee noted that revision
of the existing GMP guidelines is urgently needed and recommended that work on the revision should be started without delay.

**Guidelines for good manufacturing practice for blood establishments**

The importance of the implementation of GMP in blood establishments to improve the quality, safety and availability of plasma for fractionation as starting material was noted by the Committee. Quality assurance should cover all stages leading to the finished product, from collection (including donor selection) to storage, transport, processing, quality control and delivery of the finished product.

Blood or plasma used as a source material for the manufacture of blood products should be collected by establishments and be tested in laboratories which are subject to inspection and approved by a competent national regulatory authority.

In blood establishments, a systematic approach to ensure compliance at all steps is needed as well as assessment of compliance by a competent regulatory authority. Many countries have urged WHO to help build up the necessary technical expertise in the competent authorities.

The Committee was informed about the experiences from several workshops held in different regions of the world. It was recognized that the need for implementing quality assurance in blood establishments was generally understood. However the workshops showed that technical assistance to build up a GMP culture was urgently needed. The development and harmonization of GMP standards at a regional level and the need for training of representatives of national regulatory authorities, were also recognized as important.

The Pharmaceutical Inspection Convention/Pharmaceutical Inspection Co-operation Scheme (PIC/S) GMP guide which is already used in 28 countries is widely accepted as a basis for the establishment of GMP guidelines in different regions. This guide had been used as the working document in the regional training workshops organized by WHO for authorities, blood establishments, fractionators and inspectors. In the context of blood establishments, the PIC/S GMP guideline introduces the application of quality assurance principles in all steps involved in the collection, preparation and testing activities; supports the systematic application of donor selection criteria for each donation; reduces errors and technical problems in collection, preparation, testing and distribution; guarantees the release of products which comply with safety and quality requirements; ensures adequate documentation and full traceability for each collection and product; and provides an enabling environment for continuous improvement of the collection, preparation and testing of starting materials.
Examples of building up of regional networks among regulatory authorities and training of inspectors were presented to the Committee. At the WHO Regional Office for the Americas/Pan American Health Organization (AMRO–PAHO) Workshop on GMP, held in Buenos Aires from 28 June–2 July 2004, a three step procedure was followed:

— building up a common understanding between national regulatory authority, blood establishments and fractionators;
— building up a common language regarding the GMP standards and their interpretation; and
— specific training (e.g. for inspectors).

As an essential part of the workshop so-called mock inspections were performed in different blood establishments where essential aspects of GMP were discussed with the participants on site, thus enhancing the participants’ understanding of compliance with GMP in blood collection and processing.

The Committee agreed with a proposal for a workplan spanning several years to continue building up technical expertise in GMP for blood establishments that would include training workshops at a regional level. The Committee recommended that in the short term, workshops should be continued using the PIC/S guidelines and that WHO Guidelines be developed in the mid-term taking into account the experience from the above workshops.

**Stability of vaccines**

The Committee was informed that the Secretariat is developing guidance on regulatory expectations for stability evaluations of vaccines. This guidance is intended to assist manufacturers and national regulatory authorities in the design, conduct and evaluation of stability studies for vaccines, to complement current recommendations for particular vaccines. The need for this guidance had been identified by the Committee in 2000 and a drafting group met in 2002 to develop an outline and identify key issues. A further meeting was held in 2004 and further drafts had been developed in 2005.

The proposed scope of the guidance will cover prophylactic and therapeutic products for infectious diseases: viral and bacterial, live and non-live, combinations, rDNA products, DNA vaccines, and vectored vaccines. The guidance will cover study design issues such as testing frequency; expression of data; statistical analysis (e.g. trending); and provide links with work on the stability of reference materials.

The proposed document will include sections on accelerated stability testing and stability of intermediates, together with regulatory considerations such as stability assessment required for clinical trial approval, for licensing,
and for lot release. The document will also comment on differences and similarities in the stability testing of pharmaceuticals and biologicals and propose the use of the term accelerated stability instead of accelerated degradation. This is because for pharmaceutical products the tests look for degradation products whereas for vaccines the tests look for remaining active product. The selection of assays and other “stability indicating parameters” to detect changes in the stability profile of a vaccine will be addressed, as will an assessment of the appropriateness of thermal stability tests for lot release of live attenuated and other vaccines. Annexes addressing specific issues for some vaccines e.g. oral polio vaccine, diphtheria, tetanus (OPV), measles, D, T, whole cell pertussis (wP), meningococcal, BCG, Haemophilus influenzae type b (Hib), inactivated poliovirus (IPV), rabies, HepB etc may be included.

The Committee was informed that a further draft together with a questionnaire would be circulated to manufacturers and national regulatory authorities worldwide for comment and that a further consultation is planned for 2006 prior to the submission of the guidelines to the Committee in 2006. The Committee was asked to review the balance between general principles and specific recommendations for a vaccine or a type of vaccine (e.g., live attenuated) and the implications of having general guidelines co-existing with recommendations for specific vaccines. The Committee was also asked for advice as to whether a shelf-life (storage period) should be assigned to all stages of production (e.g., source materials seed lots/cell seeds; intermediates; harvest; bulk; final bulk).

The Committee commented that the proposed guidance was comprehensive but considered that the document should recommend definition of stability indicating parameters during vaccine development including physicochemical characteristics. The thermostability of reconstituted vaccine and storage conditions for opened vials should also be addressed. It also requested that the need for testing samples at the frequency cited in other guidelines and also at one year after the shelf-life should be considered.

In conclusion, the Committee agreed to the plan of action for these new guidelines.

**Flavivirus vaccines — regulatory expectations**

The requirements for Japanese encephalitis (JE) vaccine (inactivated) for human use (WHO Technical Report Series, No. 771, Annex 6) focus on control of production of neural tissue derived vaccine. A second guidance document (WHO Technical Report Series No. 910, 2002 Annex 3) makes specific reference to live, attenuated JE vaccine SA 14-14-2, produced on primary hamster kidney cells which is widely used in some countries. Inactivated vaccines containing Beijing 1 or SA 14-14-2 virus produced in
cell culture and a live, recombinant, Vero cell derived vaccine containing a yellow fever/ SA 14-14-2 chimeric virus are now in development.

The Committee was informed that consideration is being given to how the new JE vaccines should be evaluated, given that efficacy trials with clinical end-points were considered unsuitable for ethical and practical reasons. A WHO Consultation had therefore been held in September 2004 to discuss and achieve consensus on a scientific rationale for correlates of protection, primary end-points that may be used during clinical evaluation and additional criteria that may be used in support of registration of new JE vaccines. The consultation had concluded that neutralizing antibodies provide the best evidence that protective immunity has been established. The available evidence included passive transfer studies, linear titre protection relationship, correlation with neutralization capacity of monoclonal antibodies, protective immunity against heterologous strains and supportive data from vaccine efficacy trials. It was therefore agreed that a primary end-point of trials should be a quantitative analysis of the neutralizing antibody response against a homologous target virus, that head-to-head comparison with a registered vaccine should be undertaken and that non-inferiority to the comparator vaccine according to predefined margins based on percentage seroconversion demonstrated. A threshold antibody titre of 1 in 10 in a plaque reduction neutralization (PRNT)50 assay would be considered indicative of immunity. Additional information in support of registration would include evidence of immunological memory, qualitative analysis of neutralizing antibody response, evaluation of T cell immune response and demonstration that vaccine-induced immunity protects animals in passive protection studies.

The Committee noted the issues discussed including the strains of virus which should be used in neutralization tests and in the vaccines used in head-to-head comparisons of old and new vaccines and recommended that the Secretariat develop guidance on environmental assessment of live, recombinant (chimeric) flavivirus vaccines. It also recommended that the recommendations on inactivated JE vaccines be revised and a section on nonclinical and clinical guidance be added.

**Guidelines for acellular pertussis vaccines**

Guidelines on production and quality control of acellular pertussis (aP) vaccines were adopted by WHO in 1996. These guidelines were based on the concept of “equivalence to lots of known clinical efficacy” and for equivalence of a new vaccine to be assessed against a homologous approved aP vaccine. However, this is no longer feasible because such lots are no longer available and, moreover, there are no correlates of protection, no consensus on antigens that are important for protection and no generally accepted animal model to predict clinical efficacy.
The 1996 guidelines also recognized a need for research to standardize immunogenicity assays in mice by using homologous reference vaccine and mouse reference sera, on reliable methods for monitoring individual antigens in final bulk, on immunological markers of protection against pertussis and the identification of assays that better predict protective efficacy in humans. The guidelines also required that rigorous post-licensing monitoring for safety and effectiveness be undertaken. Agreement on studies to be conducted was reached at a WHO consultation in 2000 and the results of collaborative studies which were reported at a second consultation in 2003 indicated progress on potency and toxicity issues. The need for licensing criteria for new products and new formulations was also identified at that time.

A WHO consultation held in March 2005 recommended revision of the 1996 guidelines for acellular pertussis vaccines by means of a two-step approach. The first step is the development of licensing criteria for clinical evaluation of new vaccines and the second, the development of the scientific basis for recommendations on production and quality control, focusing on potency and toxicity tests. The issues to be considered in the clinical evaluation of vaccines include the design of the comparability studies (e.g. should they be conducted with whole-cell pertussis, or a licensed acellular pertussis vaccine; should they require tests of superiority/equivalence or non-inferiority; and what should be the duration of protection as determined in postmarketing surveillance). Additional issues relate to consideration of the acellular pertussis component as part of new combination vaccines and the interactions between components; the postmarketing surveillance required for combination vaccines; the impact of the immunization schedule and the concomitant use of vaccines.

The Committee considered that revision of the guidelines on acellular pertussis vaccine is not urgent as such vaccines are not a priority for WHO prequalification. However, as a first step, and to anticipate future trends, the development of guidance for clinical evaluation of new acellular pertussis vaccines should be initiated.

**Recommendations for bacille Calmette-Guérin vaccines**

The Committee was reminded that, despite its long history of use, several questions regarding BCG vaccines remain unresolved. These include the parameters to assess the efficacy of BCG vaccines, the significance of evolution of BCG vaccine strains, the clinical implications of genetic differences that can be detected between BCG strains, treatment for adverse events following immunization, and whether some BCG strains are antibiotic resistant. Another question is how to test BCG vaccines used as part of a novel TB vaccine prime–boost strategy. Requirements for the production and quality control of BCG vaccines were established by the
Committee (WHO Technical Report Series, No. 745, 1987) and a revision process has been initiated by the Secretariat through a Working Group. An interim report was provided to the Committee for feedback and guidance.

The proposed revision of the Recommendations would address a new method of establishing identity; relevant parameters to assess consistency of production; improved specifications for potency (currently not defined in terms of protective efficacy in an animal model); and tests for cultivable bacteria.

The Working Group also proposed that a repository of currently used strains of BCG vaccine should be established for the purpose of defining their genetic characteristics and evaluating the relevance of genetic testing for monitoring consistency of production. Further the repository may be used for the evaluation of new assays for quality control of BCG vaccines (e.g. rapid tests for viability as opposed to assaying colony forming units and in vivo protection tests). The Committee was informed that such a repository of BCG strains has been established at NIBSC.

The Committee was also reminded that it had considered a replacement for the 1st International Reference Preparation, established in 1965 on several occasions. The Working Group which met in June 2005, reviewed the intended purpose of such a replacement. They agreed that a replacement standard should be established primarily for calibration of secondary (internal or working) standards which in turn should be used for controlling the variability of viable count assays (used for estimating the numbers of live bacteria in a preparation). Other potential applications include serving as a reference for residual virulence in local reactogenicity assays, as a reference for protection assays for new BCG vaccines and as a calibrant for new tests for quality control of BCG vaccines. The Working Group noted that it was not clear if separate standards for the three sub-strains that now account for most BCG production would be required. Additional information was needed that could be obtained from a collaborative study on rapid methods for assaying viability.

The Committee agreed that scientific studies on the genetic analysis of BCG strains and standardization of potency tests should be pursued, but advised that a revision of the current Recommendations should be timed to take advantage of new data likely to be generated from the work in progress.

**International reference materials**

**Proposals for discontinuation of reference preparations**

The Committee considered proposals that the following preparations should be discontinued:
Newcastle (Hitchner B1 strain) disease vaccine, live; 1st International Reference Preparation, 1967 coded NV. A recent exercise to reconcile the WHO Catalogue with the NIBSC catalogue of international reference materials had revealed that this preparation was not transferred from the Central Veterinary Laboratories (CVL), (now the Veterinary Laboratory Agencies (VLA)), Weybridge, England to NIBSC during the exercise to transfer the custodianship of International Reference materials previously held by CVL. This was possibly because of safety considerations. The representative from the European Directorate for the Quality of Medicines (EDQM) offered to host this material if it can be determined that some of the standard still exists because some live attenuated vaccines are still manufactured and there may be a need for this material in the future. The Secretariat was therefore requested to make further efforts to find out whether the residual stock of this material is still at VLA, Weybridge and if so to transfer it to EDQM.

Scarlet fever streptococcus antitoxin, equine; 1st International Standard, 1952 coded SC. This standard was proposed for disestablishment as less than 50 ampoules are now available. Nevertheless, the Committee agreed with a suggestion that this material may of use in the future in comparisons of, or development of, assays. It was therefore proposed that the Secretariat publish the intention to discontinue this standard on their web site and if no objection is received, the proposal to discontinue this material would be reconsidered by the Committee in 2006. If there is a need to retain this material, EDQM would be prepared to hold it as a joint standard.

**Antigens and related substances**

**Yellow fever vaccine—minimum specification in International Units per 0.5-ml dose**

The First International Standard for yellow fever vaccine, NIBSC Code 99/616, was established in November 2003 with an assigned potency of $10^{4.5}$ International Units (IU) per ampoule. The WHO requirements for potency of yellow fever vaccine require that “The titre of the vaccine shall not be less than 1000 mouse median lethal dose (LD$_{50}$) or its equivalent in plaque-forming units (PFU), in the dose recommended by the manufacturer for use in humans”. However, most laboratories established the relationship between LD$_{50}$ and PFU potency tests many years ago and the relationship is not necessarily valid today. It is therefore desirable to update the minimum specification so that a minimum dose is expressed in IU.

Collaborative study data indicated that $10^1$ LD$_{50}$/0.5-ml dose may be equivalent to $10^4$IU/0.5 ml dose. However, this was found to be inappropriate
when data from assays of routine production batches were reviewed, particularly after the stability test. An alternative approach currently being investigated is to base the minimum potency on the minimum PFU or IU which results in seroconversion in clinical trials. Relevant reports identified so far indicate that a vaccine virus dose of 100 LD\textsubscript{50} from one manufacturer resulted in 100% seroconversion, but 32 LD\textsubscript{50} gave neutralizing antibody in only 19 out of 23 (87%) subjects. From the LD\textsubscript{50}/PFU/IU conversion factor of this manufacturer this suggests that 76 IU would result in 100% seroconversion. Data from a second manufacturer indicated that 1000 PFU (or 250 IU) resulted in 100% seroconversion.

As it appears unlikely that there will be sufficient data from recent clinical trials to support the establishment of a minimum dose in IU/0.5 ml, the Committee endorsed a proposal that WHO convene a meeting of yellow fever vaccine manufacturers to discuss this issue further.

**Smallpox vaccine—stability studies**

The Committee recalled that at its previous meeting a report of a collaborative study to assess the suitability of two candidate smallpox vaccines for the establishment of a replacement International Reference Preparation had been presented. The study also compared chorio-allantoic membrane (CAM) and tissue culture assays. The two candidate replacement standards had been prepared in different ways, one was a cell-culture-grown New York City Board of Health strain and the second was prepared from the Lister strain grown on calf flank. The other study samples represented a range of vaccine strains produced by a variety of methods and included the existing International Reference Preparation (IRP).

The Committee was asked to note the results of additional stability studies (WHO/BS/05.2023) on the candidate replacement preparations undertaken at the recommendation of the Committee in 2004. These studies included regular assessments of real-time stability (at –20 °C) of the candidate preparations and additional accelerated thermostability data on the two candidates generated in tests done concurrently with the current IRP at +4 °C, +20 °C and other elevated temperatures. The results of these studies indicated that at higher temperatures, 20 °C, 37 °C and 60 °C, there was no statistically significant difference between the samples, and the Arrhenius plot was uninformative. There appears to be little difference in the stability of the candidates and the current IRP.

The Committee was informed that the fill data on the two candidate materials which were both commercial vaccines were not yet available, but that demand was currently low and a delay in recommending the establishment of a replacement would not compromise availability of a reference preparation. The Committee therefore agreed that the final report including
the fill data and the recommendation on the replacement for the current IRP be submitted for the 2006 meeting of the Committee. The Committee also noted that there may be concerns regarding the import and use of the calf derived vaccine in some countries

**Poliovirus, Sabin, type 3—Neurovirulence test reference**

Neurovirulence testing is an important part of the safety testing of oral polio vaccines. The current test in primates is designed so that each monovalent bulk intended for use in OPV production is tested for neurovirulence against an appropriate reference preparation. In addition to being used in testing in primates the reference materials are also used in the transgenic mouse test. Only those bulks that pass one or other of these tests are used for vaccine production. The reference material is also included in the validation and implementation phases of the transgenic mouse test, which laboratories need to complete before using the method for quality control purposes. The above activities lead to a continuous requirement for the reference preparations.

The current WHO reference materials are +2 passages from the Sabin original seed viruses and were produced by Behringwerke, Germany, for WHO in the mid-1970s. Although there are still reasonable quantities of the WHO(SO+2)/I and WHO(SO+2)/II (the references for Types 1 and 2, respectively), the stocks of the reference for Type 3 (WHO(SO+2)/III) are very low. The Committee was informed that the properties of this reference are crucial and it is important to establish a replacement material which has similar properties to the previous reference. This is because if a replacement is too virulent, it’s use could result in batches passing that previously would have failed or, if the replacement is too attenuated, it may result in too many batches failing that previously would have passed and this could result in supply problems.

Four candidate replacements for the current WHO neurovirulence reference preparation (WHO(SO+2)/III) were produced under normal manufacturing conditions by four commercial suppliers of oral polio vaccine. Two studies have been undertaken to evaluate the properties of these materials (WHO/BS/05.2020). In the first study, four vaccines were evaluated by mutation analysis by PCR and restriction enzyme cleavage (MAPREC) analysis and in transgenic mouse tests using both a local TgMPVR21/Bx strain of mice and also in the WHO-approved TgMPVR21 mice. Two vaccines, vaccine #1 and vaccine #2, were taken forward into the second phase of a collaborative study and examined with MAPREC tests, transgenic mouse tests and monkey neurovirulence tests. As a result of this study, vaccine #1 is the most likely replacement. Consequently, 3 litres of the preparation will be distributed in ampoules, titred and the stability determined in a small
collaborative study and its behaviour relative to its intended use will be explored.

The Committee agreed that, once established, the replacement standard should be routinely used, the old one should be discontinued and remaining stocks should not be used. At the time that the replacement is established, all laboratories undertaking testing will have to establish revised C values. In addition, the Committee noted that the change in reference material will have implications for licences and changes to licences, which will have to be approved by the national regulatory authority, will be required.

The Committee noted the plans for the replacement of this reference material and requested that a full report should be submitted in 2006.

**Haemophilus influenzae type b capsular polysaccharide—1st International Standard**

A wide variety of physicochemical methods are used for the quantification of the content of the capsular polysaccharide PRP (polyribosyl ribitol phosphate; 5-d-ribitol-(1→1)-β-d-ribose-3-phosphate) in purified polysaccharides, bulk conjugates and final lots of *Haemophilus influenzae* type b (Hib) vaccines. These include phosphorus assay, ribose determination, high-performance anion exchange chromatography-pulsed amperometric detection (HPAEC-PAD) and immunochemical assays. Biological testing is carried out only to ensure safety.

A meeting to discuss WHO recommendations for the production and quality control of Hib vaccines in 1996 and a WHO/NIBSC workshop on the use of physicochemical methods for the characterization of Hib conjugate vaccines in 1998 recommended that a PRP reference preparation should be made available under the auspices of WHO. The need identified was for a reference preparation containing a known quantity of polysaccharide that can be used to cross-calibrate various methods to quantify the PRP content of the bulk saccharide, bulk conjugate and final formulations to facilitate the calibration of assays.

The candidate standard, NIBSC code 02/208, was therefore prepared. This is a freeze dried preparation of PRP unconjugated polysaccharide diluted to 5 mg/ml using sterile distilled water (DW). Ten laboratories from eight different countries participated in a collaborative study to assess its suitability; to determine its PRP content in SI units (per weight (w) basis), and to evaluate its suitability for use as a standard for PRP quantification assays (including ribose, phosphorus and HPAEC-PAD assays) of final fills and bulks of Hib vaccines (WHO/BS/05.2018). The mean PRP content as determined by the ribose assays carried out by seven of the participating laboratories
was 4.933 ± 0.267 mg/ampoule (expanded uncertainty calculated using a coverage factor of 2.45 which corresponds approximately to a 95% level of confidence).

On the basis of the results from this study, the Committee recommended that the preparation coded 02/208 should be established as the 1st International Standard for *Haemophilus influenzae* type b capsular polysaccharide polyribosyl ribitol phosphate (PRP) with an assigned unitage of 4.933 ± 0.267 mg/ampoule. The Committee requested that additional information be provided in the instructions for use concerning the use of the standard for calibration of different assays. They also recommended follow-up studies, especially to assess the value of the International Standard for assays on final fills.

**Antisera**

**Dengue virus antibody, human serum, 1st Reference Reagent**

The WHO Steering Committee on Flaviviruses identified a need for reference materials for antibodies to dengue virus as it was envisaged that the use of standards calibrated using a common unitage would facilitate the comparison of data between laboratories and of the responses to different vaccines. The availability of such materials, along with Vero cells and four serotypes of dengue virus for use in dengue neutralization tests, would facilitate the standardization of antibody assays for the detection of specific antibodies to dengue virus. These materials should be of use in the assessment of antibodies induced in vaccine trials as well as in naturally infected individuals in diagnostic laboratories.

A collaborative study divided into two phases were undertaken to assess the suitability of a freeze-dried polyvalent plasma (NIBSC code 02/186) to serve as an international reference preparation for dengue virus antibodies and to assess the variation in neutralization assays for antibodies to dengue virus (BS/WHO/05.2009).

Thirteen laboratories in six countries took part in the studies. In the first phase participants were sent coded samples which included a duplicate sample of the candidate standard and a negative control. They also received Vero cells and four dengue virus serotypes. In the second phase, participants assayed uncoded samples using the method, cells and viruses in routine use in their laboratory. Data from 32 assays on each of the four dengue virus serotypes were analysed. The 50% plaque reduction neutralization titre (PRNT<sub>50</sub>) for each sample was calculated when assayed against each virus and potencies relative to the candidate standard were calculated.

The polyvalent anti-dengue virus 1+2+3+4 serum (NIBSC code 02/186) had measurable titres against all four dengue virus serotypes tested. However,
the expression of neutralizing antibody titre of the monovalent anti-dengue sera, and a coded duplicate of 02/186, relative to the candidate standard only marginally improved the percentage geometric coefficient of variation, a measure of the variation between laboratories and assays, compared to those obtained with the expression of antibody content as a 50% plaque reduction neutralization titre.

The Committee was informed that the number of available ampoules of the polyvalent anti-dengue 1+2+3+4 preparation 02/186 is smaller than often is the case for an International Standard, as a limited amount of serum was available for processing at the time this project was initiated. Nevertheless, the Committee agreed that the preparation will be of use in facilitating the standardization of neutralization assays for antibodies to dengue virus. The Committee thus endorsed the establishment of the polyvalent anti-dengue 1+2+3+4 preparation, 02/186, as a WHO reference reagent with a unitage of 100 units for each virus so that laboratories assaying sera from recipients of vaccines currently in development can compare results. The Committee also agreed that the monovalent anti-dengue antisera preparations be issued as a reference panel but with no unitage assigned, for use in assay validation studies.

They also noted that efforts are currently under way to identify alternative sources of plasma containing antibodies to dengue serotypes 1+2+3+4.

**Japanese encephalitis virus, human serum**

At present there are no internationally available reference materials for antibodies to JE virus. The WHO Steering Committee on Flaviviruses identified the need for suitable reference materials as it was envisaged that the use of standards calibrated in International Units would facilitate the comparison of data between laboratories in the assessment of antibodies induced in vaccine trials as well as in naturally infected individuals in diagnostic laboratories.

A candidate standard designated NIBSC code 02/182 was prepared from the serum from six recipients of an internationally-available, licensed inactivated vaccine (Nakayama-NIH strain). These individuals were bled before administration of the first dose of vaccine and a control serum for use in neutralization tests is therefore also available (NIBSC code 02/184). A collaborative study was undertaken to assess the suitability of the materials to serve as international reference materials (WHO/BS/05.2008). Vero cells and three strains of JE virus were supplied to participants for use in this study. Five participants in five countries submitted data from assays on the candidate reference material and seven coded samples. The coded samples included the negative control serum and a duplicate of the candidate standard. Data from 11 assays were analysed. The expression of
neutralizing antibody titre relative to the candidate standard only marginally improved the percentage geometric coefficient of variation, a measure of the variation between laboratories and assays.

Although data are available from only a few assays, the results of the study indicate that the PRNT$_{50}$ results obtained for serum from recipients of killed vaccines, including the candidate standard, vary depending on the virus used in the neutralization tests. Thus higher PRNT$_{50}$ results are obtained when the challenge virus is homologous to the vaccine strain than when a heterologous virus is used. Potencies expressed relative to the candidate standard are therefore affected by the strain of virus used in assays. The titres obtained with the candidate material and Nakayama virus as challenge virus in the neutralizing antibody assay differ considerably from those obtained when Beijing or SA14-14-2 are used as challenge virus.

The Committee agreed that the candidate standard appears to be a strain-specific serum and is therefore unsuitable to serve as the International Standard for antibodies to JE virus. They nevertheless endorsed the view that it may be helpful for laboratories validating assays and should be made available by NIBSC for this purpose. They also noted that sera from naturally immune donors or recipients of the SA14-14-2 live vaccine may be suitable to serve as a reference material for antibodies to JE virus and that the availability of such materials is being investigated.

**Anti-human platelet antigen-1a**

Alloantibodies against human platelet antigens (HPA) are involved in neonatal alloimmune thrombocytopenia (NAIT), platelet refractoriness (PR) and post-transfusion purpura (PTP). Detection of the relevant HPA antibody is essential to the diagnosis and treatment of patients and a variety of methods have been used to detect alloantibodies against platelets. However, there is considerable variation between laboratories in the sensitivity of antibody detection tests and there is a clear need for standardization in HPA antibody detection. To date, two reference materials have been prepared by the NIBSC (anti-HPA-1a; NIBSC code 93/710 and anti-HPA-5b; NIBSC code 99/666) and both have been established as International Reference Reagents by WHO. Both are minimum potency preparations containing low titre antibodies and they are used to determine the sensitivity of tests for the respective antibodies. However, these preparations are not useful for the quantitation of antibodies, where higher titre antibodies are required for constructing a standard curve over a dilution range which reflects the variable reaction strengths found in patient samples.

Quantitation of anti-D antibodies has become routine in the monitoring of pregnancies with RhD immunization and for measuring the quantity of anti-D in anti-D preparations for prophylaxis, and international standards allow the
comparison of results between laboratories. For the former this has led to better treatment with the reliable identification of severe fetal anaemia requiring antenatal treatment with intrauterine transfusion of donor red cells. Antenatal treatment for NAIT using intra-uterine platelet transfusion is available but as it carries considerable risk it is desirable to restrict its use to the most severe cases. Currently there is no reliable predictor of severity, but the introduction of a standard for quantitation of HPA-1a antibodies will allow laboratories to measure anti-HPA-1a uniformly. This will facilitate multicentre trials and allow the relevance of anti-HPA-1a quantitation to be determined. Therefore, plasma samples containing potent anti-HPA-1a were pooled and freeze dried. In addition, three individual plasma samples were selected which had varying levels of anti-HPA-1a activity which was determined by a variety of quantitative assays using the proposed standard as a reference.

An international collaborative study which was conducted in 39 laboratories in 24 countries and showed that the anti-HPA-1a activity in three test samples could be reliably determined using the proposed standard. The assays used to determine anti-HPA-1a activity were monoclonal antibody immobilization of platelet antigens (MAIPA) assay (30 participants); commercial or in-house monoclonal antibody capture enzyme-linked immunosorbent assay (MACE ELISA) (5 participants); platelet immunofluorescence test read by flow cytometry (platelet immunofluorescence test (PIFT flow)), (3 participants).

The suitability of the candidate standard 03/152 was confirmed by the International Society for Blood Transfusion Platelet Immunology Working Party and by the International Society of Haemostasis and Thrombosis/Scientific Standardisation Committee (ISTH/SSC) Subcommittee on Platelet Immunology.

Based on the results of the collaborative study the Committee recommended that the material, coded 03/152, should be established as the 1st International Standard for quantitation of anti-HPA-1a, with an assigned unitage of 100 International Units, and that its intended use is in quantitative assays to determine the anti-HPA-1a activity in clinical samples.

The Committee also recommended that this material does not replace the earlier International Reference Reagent (93/710) which is not discontinued.

**Blood products and related substances**

**World Health Organization/International Society of Haemostasis and Thrombosis Liaison Committee report**

The activities of a new WHO/ISTH (International Society of Haemostasis and Thrombosis) liaison committee were presented. The aims of this committee are to promote the timely flow of biological standards related
to haemostasis and thrombosis through a sound peer review process in and between organizations through to final establishment by the Expert Committee on Biological Standardization; to advise the Expert Committee of priorities of various standards to be developed, replaced or discontinued as well as on new methodologies, taking into account the needs in the various WHO Regions; to hold regular discussions about information on the development and assessment of such standards; to discuss and recommend as appropriate to the ISTH/SSC and WHO/Expert Committee on Biological Standardization issues related to the preparation, assessment and publication of biological standards in the field of haemostasis and thrombosis.

The Expert Committee received a report on the first meeting of the liaison committee held on 10th August 2005 in Sydney, Australia. The following priorities were identified at the meeting: revision of WHO guidelines for thromboplastin and development and establishment of WHO reference preparations standards (e.g. a number of global measurement standards for in vitro diagnosis; platelet antibodies; and particularly replacement of the current bovine thromboplastin reference material). The liaison committee also commented on priorities proposed by NIBSC and the other collaborating centres for new and replacement reference preparations; provided expert review of standards for submission to the 2005 meeting of the Expert Committee (specifically the proposed standards for factor V plasma, factor XI plasma, prothrombin G20210A mutation, and anti-human platelet antigen 1a); and discussed ISTH/SSC publication of reports and manuscripts.

The Committee heard that it was anticipated that the liaison committee would facilitate communications between ISTH/SSC and the WHO/Expert Committee on Biological Standardization, respond to requests from WHO Regions for standards and guidelines, contribute to a continuity of approach in standards setting, guarantee and maintain records of expert peer review of all submissions to the Expert Committee, and provide a point of contact for all WHO–ISTH collaborations.

The Committee agreed with the proposed priorities for reference materials, that the continued need for the WHO bovine thromboplastin reference preparation should be reviewed and that the WHO guidelines on thromboplastins should be revised.

**Anti-A and Anti-B blood grouping reagents**

The quality of blood grouping reagents is clearly an important factor for safe blood transfusion, yet there is currently no appropriate international standardization of monoclonal anti-A or anti-B blood grouping reagents. Although WHO has International Standards for anti-A and anti-B, they were prepared many years ago and represent grouping reagents available
at that time i.e. sera from immunized donors, whereas current anti-A and anti-B grouping reagents consist of monoclonal antibody preparations. Consequently there is currently little or no demand for the existing WHO standards. WHO thus initiated development of new candidate reagents. The Committee also recalled that it had considered preliminary reports of collaborative studies to characterize two candidate standards at its 55th meeting (WHO Technical Report Series, No. 932). At that time the Committee noted that agreement on the value assignment had to be reached before the reagents could be endorsed as WHO standard reagents and the study was referred to a working group for this purpose. Advice on impact of the methodology used in the study would also be requested from the working group.

The results of a collaborative study, WHO/BS/05.2024, performed in 2005 in response to the recommendations from the Committee at its 55th meeting were presented. In this study, scientific advisors from four WHO Collaborating Centres had performed parallel titrations of the new candidate standards for anti-A and anti-B against existing reference preparations (the 2nd WHO International Standard for anti-A (code number W1001) and the 3rd International Standard for anti-B (code W1002); CBER US/FDA preparations; and British Minimum Potency Reference Preparations) to help determine minimum specifications to recommend. The specifications agreed by the scientific advisers were then communicated to the original study participants for comment. As a result of the new study the participants agreed on minimum potency specifications that were intended to ensure safety of testing, but agreed not to define an optimum specification for sensitivity of anti-A or anti-B blood grouping tests.

The Committee, taking note of the outcome of these further studies, recommended that the preparation coded 03/188 should be established as the 1st International Standard for Minimum Potency of Anti-A Blood Grouping Reagents and that a 1 in 8 dilution of 03/188 should define the recommended minimum potency specification for anti-A blood grouping reagents. The Committee recommended that a decision on the status of the 2nd International Standard for anti-A blood typing serum, code W1001, should be deferred until an evaluation is made of the demand and its current applicability. The Committee also recommended that the preparation coded 03/164 should be established as the 1st International Standard for Minimum Potency of Anti-B Blood Grouping Reagents and that a 1 in 4 dilution of 03/164 should define the recommended minimum potency specification for anti-B blood grouping reagents. The Committee likewise recommended that a decision on the status of the 3rd International Standard for anti-B blood typing serum, code W1002, should be deferred until an evaluation is made of the demand and its current applicability.
Prothrombin mutation

A non-coding mutation in the 3’ untranslated region (UTR) of the prothrombin gene G20210A (also known as 20210G>A variant of the prothrombin gene) is associated with an increased risk of arterial and venous thrombosis. Carriers of the mutation have higher plasma prothrombin concentrations than non-carriers. The mutation confers a 2- to 4-fold increased risk for venous thrombosis in the heterozygous form and a 10-fold risk in the homozygous form. Consequently, the determination of the G20210A genotype is recommended as a high priority test in the investigation of genetic thrombophilia and it has become one of the most frequent genotyping tests performed in clinical laboratories. The frequency of testing is also likely to increase as research continues on the association of pre-existing risk factors, such as a previous history of thrombosis, use of oral contraceptives or hormone replacement therapy and genetic susceptibility with venous thrombosis and air travel.

External quality assurance schemes have shown that errors in genotyping for the prothrombin mutation do occur. In 2003, results for tests of the molecular genetics of thrombophilia showed that 4.5% of laboratories had made an error when testing a sample which was heterozygous for the prothrombin mutation G20210A and that 3.1% of laboratories made an error when testing a sample containing the wild-type prothrombin gene sequence. These errors can have a significant and long-lasting impact on the patient, particularly because genotyping tests are usually carried out only once on any one patient. This is in contrast to many other pathology tests which are often carried out on several occasions, allowing errors to be identified.

Forty-five laboratories participated in an international collaborative study to assess the suitability of a panel of three gDNA samples as the 1st International Genetic Reference Panel for Prothrombin Mutation G20210A, Human gDNA. The participants employing a total of 22 methods evaluated the panel against their in-house controls which were samples from known patient and commercial controls.

The results have shown that the panel of three gDNA samples evaluated in this international multicentre study are suitable as reference materials for laboratories carrying out genotyping for prothrombin mutation G20210A. Therefore the Committee endorsed the three human gDNA preparations 04/194 (Prothrombin wild type), 04/174 (Prothrombin Mutation 20210 Heterozygote), 04/196 (Prothrombin Mutation 20210 Homozygote) as the 1st International Genetic Reference Panel for Prothrombin Mutation G20210A, Human gDNA. A panel of materials, comprising one of each of the above preparations, will be made available from NIBSC with the code number 05/130.
Vitamin B12 and folate in human serum

The assay of the vitamins B12 and folate in blood is the current routine procedure for determining deficiency of these vitamins. Deficiency can result in a number of clinical conditions including megaloblastic and pernicious anaemia.

The 1st International Reference Reagent for human serum vitamin B12 (81/563) was established by WHO in 1992. However, the preparation has since been found to be positive for anti-hepatitis C virus (HCV) and HCV RNA. Also, it was calibrated using a microbiological assay (Euglena assay) which has since been replaced by automated assay systems. For these reasons, it was decided to replace the 1st International Reference Reagents and assign a value to the replacement preparation, independently of the value assigned to the previous standard, using current methodology.

The aim of the study (WHO/BS/05.2025) was therefore to evaluate a batch of lyophilized serum for folate and B12 content using current methods, including candidate reference methods based on mass spectrometry for the specific determination of 5-methyltetrahydrofolate (5MeTHF) and other folate forms in serum or plasma. The candidate International Standard for B12 and serum folate, 03/178, was assayed using a wide range of methods in 24 laboratories in seven countries.

The Committee recommended that the preparation, coded 03/178, should be established as the 1st International Standard for serum folate, with a unitage of 12.1 nmol/l (5.33 ng/ml) of folate and the 2nd International Standard for vitamin B12 with an assigned unitage of 480 pg/ml, when the freeze-dried contents of the ampoule are reconstituted in 1 ml of distilled water. The Committee also recommended that a corrected report be submitted to WHO showing the vitamin B12 value in pg/ml, not ng/ml as stated in BS.05/2025. Finally the Committee recommended that the assigned vitamin B12 value of 480 pg/ml should be re-evaluated when a reference method has been established.

Blood coagulation factor V (plasma) human

Estimation of factor V clotting activity (FV:C) in human blood plasma is performed for several reasons. These include the diagnosis of very rare congenital bleeding disorders such as FV deficiency (“parahaemophilia”) and combined FV/FVIII deficiency, as well as investigations into coagulation disorders linked to liver disease. The need to review the management of rare bleeding disorders, including improved standardization of diagnostic coagulation tests, has been highlighted. Factor V:C estimation is also a requirement for the quality control of the therapeutic product, virus-inactivated fresh frozen human plasma, as described in the European
Pharmacopoeia. Despite the existence of several commercial reference plasmas with assigned values there is currently no internationally accepted unit (IU).

An international collaborative study involving 22 laboratories in 11 different countries was undertaken to calibrate a proposed 1st International Standard for Factor V clotting activity (WHO/BS/05.2007). Most estimates (21/23) were obtained using thromboplastin-based methods rather than activated partial-thromboplastin time (APTT)-based methods. There was very good agreement between estimates with inter-laboratory variability of 3.55% and a range of estimates from 110–124% of the proposed assigned value of the proposed standard. There was also good agreement between results from APTT- and thromboplastin-based methods indicating that the proposed standard will be suitable for use with both methodologies. Factor V is recognized as a labile coagulation factor and can suffer loss of activity on storage in the liquid state or through cycles of freezing and thawing. Accelerated degradation studies in three laboratories indicated that the proposed standard is extremely stable and suitable for prolonged use.

On the basis of the results of the study the Committee endorsed the WHO 1st International Standard Factor V Plasma (03/116) with an assigned value of 0.74 IU Factor V:C per ampoule.

Blood coagulation factor XI (plasma) human

Factor XI deficiency is inherited as an autosomal recessive bleeding disorder known as haemophilia C. Factor XI deficiency is most frequently observed in Ashkenazi Jews and Iraqi Jews. Patients with factor XI deficiency have also been reported in African-American, Arab, Chinese, English, German, Indian, Iranian, Italian, Japanese, Korean, Portuguese and Swedish populations.

There are two plasma derived concentrates available for the replacement therapy and these concentrates are calibrated against different plasma pools. Currently, no WHO International Standard is available for calibration of FXI activity and laboratories employ locally collected normal plasma pools or commercially available frozen or freeze-dried plasma as references. A common standard is required to improve inter-laboratory agreement for measurement of FXI activity.

Twenty-seven laboratories from 11 countries took part in a collaborative study to assign a potency value to a proposed WHO 1st International Standard for Blood Coagulation Factor XI, Plasma, Human (04/102) and also to calibrate the Scientific Standardization Committee (SSC) secondary plasma standard Lot#3 for factor XI activity (WHO/BS/05.2017). The main aim of this study was to assign an internationally agreed potency value in IU to a batch of pooled normal plasma relative to locally collected normal plasma pools.
On the basis of the results, the Committee endorsed Plasma, Human (04/102) as the WHO 1st International Standard for FXI with an assigned value estimated against pools of both fresh and frozen normal plasma of 0.86 IU/ampoule. This standard is intended for use in the in vitro diagnostics area and will be available for the calibration of commercial reference plasmas and of secondary and in-house standards.

**Thromboplastin, rabbit, plain**

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 necessary to convert PT results into an International Normalized Ratio (INR). Observations from users show that the calibration of a given thromboplastin is generally more precise when it is performed against an International Standard of similar composition, and from the same species, and is one of the reasons that WHO maintains thromboplastin International Standards from different species.

Since stocks of the existing International Standard coded RBT/90 (rabbit, plain) are exhausted, a preparation of rabbit brain thromboplastin coded 04/162 was calibrated in an international collaborative study organized and carried out under the auspices of the Subcommittee on Control of Anticoagulation of the Scientific and Standardization Committee (SSC) of the International Society on Thrombosis and Haemostasis (ISTH). Since RBT/90 was no longer available, it could not be used for the calibration of the replacement candidate. Therefore the study (WHO/BS/05.2029) included five secondary reference materials for rabbit thromboplastins previously calibrated against RBT/90 instead. Twenty-two laboratories in 13 countries participated in the study. Most used the manual (tilt tube) technique for PT testing.

Based on the results of the study the Committee endorsed preparation 04/162 as the 3rd WHO International Standard for Thromboplastins, rabbit, (plain) with an assigned International Sensitivity Index value, calibrated against the five existing International Standards, of 1.15.

**Cytokines, growth factors and endocrinological substances**

**Vascular endothelial growth factor, VEGF\(_{165}\) - 1st Reference Reagent**

Vascular endothelial growth factor (VEGF, also known as VEGF-A) belongs to a gene family that includes VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF). Through its actions on endothelial cells, VEGF induces vasculogenesis (differentiation of endothelial cell precursors), angiogenesis (growth of blood vessels), survival of immature blood vessels (vascular
maintenance) and lymphangiogenesis (growth of lymphatic vessels). It is a potent vascular permeability factor in vivo and is a chemo-attractant for monocytes and endothelial cells. VEGFs are under investigation, by administration of the protein or as a gene therapy product, for stimulation of angiogenesis in ischaemic disease and lymphangiogenesis in treatment of lymphoedema. VEGF expression is elevated in some types of cancer, and measurement of VEGF levels for diagnosis and prognosis is under investigation. Inhibition of VEGF activity has wide potential therapeutic application in the treatment of various cancers and other conditions involving pathological development of blood vessels.

The availability of a reference standard for VEGF could facilitate measurement of the potency and stability of therapeutic preparations of VEGF and inhibitors, and measurement of VEGF levels for diagnosis.

Preparations of human sequence recombinant vascular endothelial growth factor-165 (VEGF$_{165}$) synthesized in *Escherichia coli* were formulated and lyophilized at NIBSC. The first preparation, 01/424, was non-homogeneous in appearance, and following evaluation at NIBSC, was distributed as a NIBSC research reagent. A second preparation, 02/286, was lyophilized in a different formulation and evaluated in a collaborative study for its suitability to serve as a reference standard, and compared with preparation 01/424, by five laboratories using in vitro bioassays or immunoassays (WHO/BS/05.2028). The candidate standard was assessed for stability using thermally accelerated degradation samples. None of these samples showed any evidence of loss of activity at any of the elevated temperatures, including the relatively high temperatures of 37 °C and 45 °C.

The Committee endorsed the proposal that the preparation 02/286 be established as the WHO reference reagent for VEGF$_{165}$ with an assigned unitage of 13 000 units per ampoule. To assist laboratories currently using mass units for VEGF$_{165}$ preparation 02/286 could be considered to contain approximately 13 µg VEGF$_{165}$ per ampoule, based on the predicted content from the manufacturer’s stated concentration and the results of this study, and would be noted in the memorandum with instructions for use. The Committee was also informed that preparation 01/424 will continue to be made available as an NIBSC research reagent for at least 2 years after the establishment of 02/286 as a WHO reference reagent, to provide continuity for laboratories currently using this preparation and to permit them to make a direct comparison of the two preparations in their own assay systems.

**Keratinocyte growth factor—1st Reference Reagent**

Keratinocyte growth factor (KGF), also known as fibroblast growth factor-7 (FGF-7) is a single chain secreted glycoprotein with target specificity
restricted to epithelial cells in which it stimulates proliferation, differentiation and migration. KGF is under investigation for a number of therapeutic applications involving stimulation of proliferation of epithelial cells. Measurement of endogenous KGF levels may have applications in diagnosis and prognosis. A modified form of recombinant KGF synthesized in E. coli, is licensed for the reduction of incidence and severity of oral mucositis in cancer patients receiving a high dose of chemotherapy and radiation therapy. The availability of a reference preparation would facilitate inter-laboratory comparison of potency and immunoassay measurements of KGF and stability studies of therapeutic formulations.

Two preparations of recombinant KGF synthesized in E. coli, one consisting of the normal mature 163 amino acid sequence of human KGF, and the other of a modified sequence lacking the 23 N-terminal amino acids were tested by five laboratories using in vitro bioassays or immunoassays (WHO/BS/05.2027). A preparation which contained no carrier protein was included in some studies. The different preparations were clearly distinguished by the different assay systems, demonstrating the need for separate reference preparations in terms of which bioactivity can be expressed, and which could be used to assess assay specificity. Each of the candidate preparations was judged sufficiently stable to serve as a reference standard, although the Committee noted that some additional studies on reconstituted material will be undertaken so that appropriate advice can be included in the information for users.

The Committee endorsed the proposal that the preparation coded 03/150 be established as the WHO reference reagent for human KGF, with an assigned unitage of 4000 units of KGF per ampoule, and that the preparation coded 03/148 be established as the WHO reference reagent for human KGF (24-163), with an assigned unitage of 9000 units of KGF(24-163) per ampoule.

In addition, it was agreed that in order to assist laboratories currently using mass units for KGF, 03/150 could be considered to contain approximately 4 µg KGF per ampoule and 03/148 could be considered to contain approximately 9 µg KGF (24-163) per ampoule based on the content predicted from the manufacturer’s stated concentration and the results of this study, as would be noted in the memorandum with instructions for use. The Committee noted that freezing and thawing of reconstituted solutions can alter their properties and that freezing and reuse is therefore not recommended.

**Measurement of relative potencies of thermal degradation samples of the WHO International Standard of interferon alpha 2b (ifn-α2b), 95/566**

Several International Standards for different human interferon (IFN) types have been established which serve as primary calibrators of bioassays for
secondary working reference preparations of the same IFN. An important aspect of their suitability to serve as primary calibrators is the requirement for long-term thermal stability of IFN activity at the repository storage temperature of ampoules. It is often not possible to study thermal stability in real time as normally an International Standard would be established 2–4 years after its preparation and evaluation by collaborative study, a period of time usually too short to allow the evaluation of potential losses of activity in ampouled material held at the recommended storage temperature of –20 °C. Thermal stability is therefore assessed in accelerated thermal degradation studies. Nevertheless, since most IFNs are very stable, even accelerated degradation studies may require protracted storage of ampoules—longer than the limited time-frame before establishment of an International Standard—for the procurement of data that accurately predicts rate of loss of activity at the recommended storage temperature. For example, accelerated thermal degradation studies on the current WHO International Standard of IFN-α were carried out 0.9 to 1.7 years after manufacture and, because there was no indication of thermally instability at –20 °C, the materials were considered to be suitable for establishment (WHO/BS/99.1911).

The continued availability of accelerated thermal degradation samples after longer storage times provided an opportunity to re-assess whether the thermal stability of activity confirmed the expected robust stability of IFN-α at –20 °C. The activity of the 2nd WHO International Standard for IFN-α2b (NIBSC code 95/566) stored for approximately 9 years has therefore been examined (WHO/BS/05.2006). Two assay systems were used—an antiviral assay and a reporter gene assay. The accuracy and precision of these assays were compared to determine which method provided the most reliable data for assessing the thermal stability of activity. Two sets of ampoules of the standard coded 95/566 were retrieved from storage at temperatures of –150 °C, –70 °C, –20 °C, 4 °C, 20 °C, 37 °C, 45 °C and 56 °C on 12 July 2004, approximately 9 years from the fill-date of 29 May 1995. One set was retained at NIBSC for testing in antiviral assays, the other set was sent to The National Institute of Chemistry, Ljubljana, Slovenia, for testing in reporter gene assays.

The results of this study provided strong evidence that the activity of the WHO International Standard for IFN-α2b contained in ampoules of 95/566, is extremely stable at the customary storage temperature of –20 °C. They also confirm the expected long-term thermal stability of this preparation and its suitability to serve as the International Standard for the duration of its usage (to near exhaustion of the stock of ampoules), i.e. approximately 25 years. Comparison of the results derived from (a) antiviral assays and (b) reporter gene assays showed they were very similar for this IFN-α2b preparation. The lower intra-assay variation of estimates from reporter
gene assays suggests they could provide a valuable alternative method for evaluating the thermal degradation studies of other WHO International Standards for type I IFNs, e.g. IFN-β.

The Committee noted the contents of the report and also encouraged publication of this, and other, examples of long-term studies of the stability of biological reference materials.

**Diagnostic reagents**

**Reference materials for in vitro diagnostic devices**

The Committee was provided with an overview of the current activities of the International Federation of Clinical Chemists (IFCC) related to standardization in laboratory medicine. It was noted that the Scientific Division of the IFCC through its Committees and Working Groups is involved in more than 15 projects on development of reference materials and/or reference measurement procedures following the criteria defined in International Standards Organization (ISO) Standards 15193 and 15194. The IFCC contributes to the development of the concept of reference measurement laboratories and is involved in developing a process for review of reference measurement services and criteria of recognition of reference laboratories in laboratory medicine. Through its Committee on Traceability in Laboratory Medicine, the IFCC establishes and implements external quality assessment schemes (EQAS) for reference laboratories for monitoring competence through comparative measurement campaigns. Further, the IFCC is a major partner and founding member of the Joint Committee on Traceability in Laboratory Medicine (JCTLM) which has established two major Working Groups. The first is involved in evaluating nominated reference measurement procedures and reference materials by various national and international organizations, and publishes a list of reference materials and reference measurement procedures that meet ISO criteria. These lists are available for consultation through the IFCC web site. The second Working Group on reference measurement laboratories is currently working on defining a process for review of reference measurement services from laboratories following ISO 17025/15195 Standards. Finally a list of current IFCC standardization activities was provided to the Committee.

**HIV-1 RNA nucleic acid amplification test assays**

The 1st International Standard for HIV-1 RNA for HIV-1 NAT assays, code 97/656, was established by the Expert Committee in 1999 and has since been extensively used for assay validation and calibration and to calibrate secondary standards and working reagents. The 1st International Standard was prepared from a subtype B (V3) HIV-1 polymerase chain reaction (PCR)-
positive, antibody-negative plasmapheresis donation from the USA that was
diluted in screened defibrinated plasma and freeze-dried. Approximately
2300 vials were prepared, each containing, when reconstituted, a volume
of 1 ml. This material was evaluated in an international collaborative study
together with a different batch of freeze-dried diluted HIV and a liquid HIV
preparation. All three preparations gave results that were tightly grouped,
with little difference between the results from different laboratories or
those obtained by the use of different assays. The Committee designated
the preparation 97/656 as the 1st International Standard and assigned it a
potency of $5.0 \log_{10} (100\,000)\text{ IU per vial}$.

In 2003, it was decided to evaluate a replacement International Standard
as the number of vials remaining had decreased to ~1000 (with a mean
usage of 240 vials per annum). At current usage rates supplies would be
exhausted in 3–4 years and it was also discovered that the current standard
was contaminated with hepatitis B virus (HBV) DNA which, although not
a problem for single marker NAT assays, did present a problem for the
increasing number of multiplex NAT assays being developed and coming
into use. This was discussed at the 16th International Scientific Working
Group on the Standardisation of Genome Amplification Techniques (SoGAT)
in July 2003 where it was agreed that a replacement standard was needed
and that the second freeze-dried candidate from the first collaborative study
should be re-evaluated in a new collaborative study.

The objectives of the present collaborative study (WHO/BS/05.xxxx)
were to establish a replacement WHO International Standard for HIV-1
RNA NAT assays, to determine the real time stability of the current and
candidate preparations over the period since they had been freeze-dried
and to establish the relative potency of the candidate replacement standard
(97/650) against the WHO 1st International Standard (97/656) to ensure
continuity of the IU.

The Committee, taking note of the report, endorsed the establishment of
candidate material 97/650 as the 2nd International Standard for HIV-1 RNA
NAT assays and assigned a unitage of $5.56 \log_{10} \text{ IU/vial}$. This is equivalent
to $5.56 \log_{10} \text{ IU/ml}$ when a freeze-dried vial is reconstituted with 1 ml
purified water.

**Anti-HIV tests**

The human immunodeficiency virus (HIV), a retrovirus that is the cause of
AIDS, exhibits considerable sequence diversity which forms the basis of its
genetic subtyping. A number of groups and subtypes are recognized including
HIV-1 groups M (subtypes A–K), N and O and HIV-2. Despite good progress
having been made on genotyping HIV through sequence diversity, little
progress has been made on the serotyping of HIV. Infected individuals mount
a strong humoral immune response, starting about 3 weeks after infection, and antibodies that react in enzyme immunoassays (EIAs) are produced to all the main structural proteins. Functional immune responses, such as the development of virus-neutralizing antibodies, also occur, although they may take a considerable time to develop and titres are usually low. Attempts to show a relationship between virus-neutralization serotypes and sequence-based genotypes have to date been largely unsuccessful.

During 1994, it was discovered that certain screening tests, particularly those based on synthetic peptides, were unable to detect infection with some of the more diverse outlier group O viruses. This resulted in the withdrawal of a number of assays and, over the subsequent years, most assays were modified to broaden their range and enable them to better detect antibodies to outlier viruses and to enhance their sensitivity.

In 1998, a meeting of the WHO Working Group on Reference Preparations for Testing HBsAg, anti-HCV and anti-HIV Diagnostic Kits recommended that an anti-HIV reference panel consisting of a panel of plasma samples representing the major groups and subtypes of HIV to reflect HIV diversity around the world should be established. Such a panel would:

• assist users of serological tests in different regions of the world in determining that the test they use is able to detect antibodies to the subtype(s) prevalent in their region;
• help kit manufacturers and others to demonstrate the capability of their assays to detect antibodies to all the main HIV subtypes and groups; and
• be of value in assessing the analytical sensitivity of assays with regard to their ability to detect diluted samples.

A pilot study was performed the results of which were used to select samples for inclusion in a definitive anti-HIV reference panel (code 02/210). The panel consists of solvent-detergent treated anti-HIV-positive human plasma samples that have been diluted 1 in 40 in anti-HIV-negative human serum and freeze-dried. The anti-HIV-positive plasma samples were derived from individuals infected with HIV-1 subtypes A, B, C and E, HIV-1 group O and HIV-2 and were selected using the criteria of type, subtype and group, titre, Western blot profile and availability.

Thirteen laboratories from around the world took part in the collaborative study. Participants were requested to test the reference panel for anti-HIV in as wide a range of assays as possible. Where appropriate, it was recommended that serial dilutions were performed to facilitate the comparison of analytical sensitivity between assays. For assays such as Western blots, assessment of their performance undiluted was considered sufficient. The intention was that a dossier detailing the performance of reference panel samples in a range of tests would be provided with the panel to enable the recipient
to compare the performance of the panel with kits of their choice in their own laboratory with the results from elsewhere. Assigning a unitage to the samples was not considered appropriate.

The Committee was informed that 24 datasets had been received. Preliminary results show that the anti-HIV-negative human serum sample was negative in all assays and that all HIV-positive samples were detected in all assays, with the exceptions that an anti-HIV-2 EIA failed to detect some HIV-1 samples and a particle agglutination test failed to detect the group O sample. Once all data have been included in the analysis and the results discussed with the participants, a full report will be submitted to the Committee.

The Committee noted the progress that was being made and requested that a full report be submitted at its 2006 meeting.
Annex 1

Guidelines for assuring the quality and nonclinical safety evaluation of DNA vaccines

This document provides information and guidance to national regulatory authorities and vaccine manufacturers concerning the characteristics, production, control and nonclinical development of DNA vaccines. The text is written in the form of Guidelines instead of Recommendations because further work is still needed to develop and standardize appropriate methods and criteria that will assure the consistent quality, safety and stability of these vaccines. Guidelines allow greater flexibility than Recommendations with respect to expected future developments in the field and indicate present deficiencies.

1. Introduction
2. Scope of the guidelines
3. Definitions
4. General manufacturing considerations
5. Manufacture and control of bulk purified plasmid (drug substance)
   5.1 General information
   5.2 Manufacture
   5.3 Characterization
   5.4 Control of bulk purified plasmid
   5.5 Reference standards or materials
   5.6 Stability
6. Manufacture and control of final formulated vaccine (drug product)
   6.1 Composition
   6.2 Manufacture
   6.3 Control of materials
   6.4 Control of final formulated vaccine
   6.5 Reference materials
   6.6 Stability
7. Nonclinical safety evaluation
   7.1 Introduction
   7.2 General considerations on safety
   7.3 Considerations on the nonclinical safety programme
   7.4 Toxicity studies
   7.5 Immunogenicity studies
   7.6 Biodistribution, persistence and integration
   7.7 Genotoxicity
   7.8 Developmental and reproductive toxicity

Authors
Acknowledgements
References
1. **Introduction**

Vaccination involves priming the immune system of a host with an infectious agent or components of an infectious agent modified in a manner to ensure that the vaccine does not cause any harm or disease to the host, but ensures that when the host is confronted with that infectious agent, its immune system can respond adequately control the invading organism before it causes any ill effect. For over a hundred years immunization has been achieved by one of two basic approaches:

- introducing into the host, specific antigens against which the immune system will react directly; or
- introducing attenuated living organisms which replicate within the host without causing disease and synthesize the appropriate antigens which subsequently prime the immune system.

Since the early 1990s a radically new approach to vaccination has been actively and vigorously developed. This involves the direct introduction of plasmid DNA containing the gene encoding the antigen against which an immune response is sought into appropriate host tissues and the in situ production of the target antigen(s). This approach offers a combination of potential advantages over the more traditional approaches, including the stimulation of both B and T cell responses, improved stability of the vaccine, absence of any infectious agents and the relative ease of large scale manufacture. Many scientific publications address the potential of DNA vaccination and immune responses in animal models have been obtained using genes from a variety of infectious agents including influenza virus, hepatitis B virus, human immunodeficiency virus, rabies virus, lymphocytic choriomeningitis virus, West Nile virus, malaria and mycoplasma. In many cases protection from disease in animal models has also been demonstrated and many aspects of the immune response generated by the injection of plasmid DNA vaccines have been revealed although much remains to be understood. The value and advantages of DNA vaccines will be assessed on a case-by-case basis and their applicability will depend on the nature of the organism being immunized against, the nature of the antigen and the type of immune response required for protection.

DNA vaccines progressed rapidly into phase I clinical trials. However, the immune responses observed in animal models have generally not been reproduced in humans and many approaches have been and are being followed to enhance the human immune response. These approaches function in different ways such as in enhanced uptake, stability of expression, modulation of the immune response, or in adjuvanting, and include:

- complexing the DNA with polymers (enhances uptake, improves stability);
• encapsulating the DNA on or within microparticles (assists uptake, presentation and stability);
• optimization of the codon usage of the gene encoding the antigen of interest (enhances expression);
• encoding a variety of T cell epitopes either instead of or in addition to a full size protein antigen (modulates the immune response by targeting T cell stimulation);
• optimizing administration, e.g. by particle-mediated delivery (gene gun) or electroporation (enhances uptake, modulates immune response);
• route of administration, e.g. mucosal versus parenteral (modulates the immune response);
• boosting with viral vectors or protein antigen following an initial priming with plasmid DNA (improves immune response); and
• co-administration of DNA encoding an immune stimulatory molecule (molecular adjuvant), e.g. a cytokine (improves immune response, modulates the immune response).

Other approaches may also be under development now or in the future. The above approaches to enhancing the efficacy of a DNA vaccine may raise specific safety concerns and these should be addressed in appropriate nonclinical safety testing.

DNA vaccines are also being developed for veterinary use and efficacy in animal target species is being observed in some trials. Potentially protective immune responses are being observed against many infectious agents in several target species including fish, and companion and farm animals. A DNA vaccine against West Nile virus for use in horses was first licensed in the USA in 2005. Although the quality and safety considerations for vaccines for veterinary use differ from those for human use, experience with veterinary DNA vaccines can provide valuable information for the control and use of human DNA vaccines.

These guidelines concentrate on the quality control and on nonclinical testing of vaccines based on bacterial plasmid DNA intended for use in humans. The purpose of this document is to provide guidance on:

• appropriate methods for the control of the manufacture and characterization of plasmid DNA vaccines;
• appropriate approaches for the nonclinical testing of plasmid DNA vaccines; and
• information specific to plasmid DNA vaccines that should be included in submissions by manufacturers to national regulatory authorities in support of applications for the authorization of clinical trials and for marketing.

The development and application of DNA vaccines continues to evolve. Since these guidelines were first adopted in 1996 (1), many clinical trials of
DNA vaccines have taken place and much experience in their manufacture and control has accrued. This revision reflects the experience gained, especially in relation to the data derived from nonclinical safety testing and the concerns expressed in the first version of these guidelines. The control of these vaccines should continue to be approached in a flexible manner to enable further modifications as more experience is gained in their production and use. The intention is to provide a scientifically sound basis for the manufacture and control of these vaccines for use in humans so as to ensure their consistent safety and efficacy. Individual countries may wish to use this document to develop their own national guidelines for DNA vaccines.

2. **Scope of the guidelines**

This document provides guidance on quality and nonclinical aspects of DNA vaccines intended for use in humans.

The active constituent of a DNA vaccine is a plasmid molecule that contains the gene for a component of a pathogenic organism under the control of a mammalian expression system, and possesses DNA sequences necessary for replication and selection in bacteria. Although a vaccine is generally defined as a biological medicinal product for the prophylaxis of infectious disease, “DNA vaccines” are also being developed for therapeutic use, either against infectious disease or for other diseases such as cancer. In the case of cancer, the relevant gene often has a human origin (e.g. a cytokine) rather than a microbiological origin. DNA vaccines against infectious disease may also contain plasmids expressing genes of human origin which act as molecular adjuvants. It is clear that the manufacture and quality control of plasmid DNA for any of the above indications will be essentially identical and consequently, these guidelines are applicable to DNA vaccines for therapeutic as well as prophylactic use. The detailed design of relevant nonclinical safety testing should take into account the proposed use of the DNA vaccine and the risk–benefit situation.

The guidelines cover DNA vaccines regardless of their method of delivery. The quality section of these guidelines will be applicable to DNA plasmids that contain mammalian viral replicons; however, different requirements may apply to nonclinical testing of such products and the present guidelines do not address these. Similarly, many aspects of the guidelines may be applicable to vaccines based on RNA, although again, different requirements are likely to apply especially for nonclinical safety testing for these types of vaccine. Plasmid DNA vaccines for use in gene therapy, DNA vaccines derived in eukaryotic cells, vaccines in which a bacterial cell acts as a carrier for a plasmid DNA encoding a relevant antigen and nucleic acid vaccines
made entirely by chemical means such as synthetic oligonucleotides are all outside the scope of these guidelines.

The quality section addresses the control of the bulk purified plasmid (drug substance) including control of the manufacturing process and the starting materials, characterization of the purified plasmid and control of the final formulated vaccine (drug product) including formulation, the control of materials used in formulation and characterization of the final vaccine.

The nonclinical safety section addresses the approaches to be followed during testing of the vaccine prior to clinical use. The background information provided in the quality section should be considered when designing appropriate nonclinical safety studies.

In general, recommendations in these guidelines are relevant to the product at the time of application for marketing approval. Some relevant information is provided with respect to products in development in these guidelines; otherwise, the respective national regulatory authority should be consulted prior to clinical development on a case-by-case basis (2, 3).

The control and nonclinical testing of each vaccine should be considered individually and any special features should be taken into account. Furthermore, the application of these guidelines to a particular vaccine should reflect its intended clinical use. Thus, different criteria will apply to a vaccine that is to be used prophylactically in healthy children universally, than to one that is to be used therapeutically for treating a life-threatening condition.

3. Definitions

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

*Bulk purified plasmid (drug substance).* The purified plasmid before final formulation. It is obtained from one or more bulk harvests, and is kept in one or more containers designated as a single homogeneous production lot and used in the preparation of the final dosage form (drug product).

*Final formulated vaccine (drug product).* The finished formulated vaccine product. It may be freeze-dried and/or contain excipients and/or adjuvants.

*Master cell bank (MCB).* A homogeneous suspension of bacterial cells, already transformed by the plasmid containing the desired gene, dispensed in aliquots into individual containers for storage. All containers are treated identically during storage and, once removed from it, are not returned to the cell bank.

*Plasmid.* A circular, extrachromosomal bacterial DNA element which undergoes autonomous replication in bacterial cells. It usually carries a
few genes, some of which confer resistance to various antibiotics; such resistance is often used to discriminate between organisms that contain the plasmid and those that do not.

**Working cell bank (WCB).** A homogeneous suspension of bacterial cells derived from a single vial of the master cell bank dispensed in aliquots into individual containers for storage. All containers are treated identically and, once removed from storage, are not returned to the cell bank. Typically, a single or a defined number of aliquots is used to manufacture a batch of vaccine. In some cases, a working cell bank may not be established and vaccine manufacture may begin from an aliquot of the master cell bank.

4. **General manufacturing considerations**

Plasmid DNA vaccines are considered to be similar to bacterial and viral vaccines produced by traditional methods, where adequate control of the starting materials and manufacturing process is just as important as that of the product. The guidelines therefore place considerable emphasis on “in-process” controls for assuring the safety and effectiveness of the vaccine as well as on comprehensive characterization of the vaccine itself.

The general manufacturing requirements contained in good manufacturing practices (GMP) for pharmaceutical (4) and biological (5) products will apply to plasmid DNA vaccines. DNA vaccines for use in clinical trials should also be prepared under GMP conditions. Appropriate attention therefore needs to be given to the quality of all reagents used in production, including the components of fermentation media. Many of the general requirements for the quality control of biological products, such as tests for potency, endotoxin, stability and sterility, also apply to DNA vaccines.

It is recognized that the level of detail required by a regulatory agency increases as product development proceeds. During the initial phases of clinical development, the information contained in a clinical trial application should be adequate to allow an assessment of the safety risks from the manufacturing process. This would include, for example, testing of the cell banks for identity, identification and specifications for all materials used in the process, assessment of risks from biologically-sourced materials, a brief description of the process and tests, results of testing of the clinical trial material and preliminary stability of the drug product.

For late-stage clinical trials the level of detail would increase to include preliminary evidence of consistency of manufacture and validation.

Changes made to the product composition (e.g. addition of adjuvant or preservatives) or manufacture (process, site or scale) during the development of clinical and postapproval manufacturing lots may have a significant
impact on safety and/or efficacy. Any change in the production of a DNA vaccine places a responsibility on the manufacturer to show that the product is equivalent to that used in preclinical studies or earlier stage clinical trials. Such changes should be evaluated on a case-by-case basis to determine what supporting data should be provided to show comparability of the modified version with the previous one.

5. Manufacture and control of bulk purified plasmid (drug substance)

5.1 General information

A brief overview of the development and manufacture of the product including a justification for the selection of the gene(s) of interest, the rationale for the development of the product and the proposed route of administration should be provided.

5.2 Manufacture

5.2.1 Description of manufacturing process and process controls

Information should be provided on the manufacturing process, which typically starts with a vial of the cell bank, and includes fermentation, harvest, purification, filling into bulk containers and storage.

A flow chart should be provided illustrating the manufacturing steps from the cell bank up to the drug substance. The chart should include all steps (i.e. unit operations), identification of materials, major equipment and in-process controls.

A description of each process step in the flow chart should be provided and each step justified. Information should be included on, for example, scale; culture media, buffers and other additives; major equipment; and process controls, including in-process tests and operational parameters with acceptance criteria. Information on procedures used to transfer material between steps, equipment, areas, and buildings, as appropriate, and shipping and storage conditions should be provided by the time of application for marketing authorization.

Data on the consistency of fermentation conditions, culture growth and cell and plasmid yield should be presented. Criteria for the rejection of culture lots should be established. The maximum level of cell growth and scale to be permitted during production should be specified, based on information on the stability of the host-cell/plasmid system up to and beyond the level of fermentation used in production by the time of application for marketing authorization.
The characteristics of bacterial cells/plasmids at the end of fermentation should be investigated. This investigation should include, as a minimum, plasmid copy number and restriction enzyme mapping.

For materials such as filter membranes and chromatography resins, information on conditions of use should be provided. Filter membranes and chromatography resins should be dedicated to a single product. In the event that a column or filter is re-used for a single product, conditions of re-use should be provided.

The methods used for harvesting, extraction and purification should be described in detail and justified. The process should be designed to remove process- and host-related contaminants, such as endotoxin, host RNA, chromosomal DNA and any other materials considered undesirable in the final product.

5.2.2 Control of materials

The materials used in the manufacture of the drug substance (e.g. raw materials, starting materials, solvents, reagents and catalysts) should be listed and information given on where each material is used in the process. Information on the quality and control of these materials should be provided. Reference to internationally accepted pharmacopoeias or details on the specifications should be provided.

5.2.2.1 Control of source and starting materials of biological or animal origin

Information regarding the source, manufacture and characterization of all biologically-sourced materials or materials that may have used biological materials during manufacture should be provided. Summaries of the viral safety information should be provided including appropriate certification where applicable.

5.2.2.2 Source, history and generation of the host cell

Information should be provided on the bacterial host cell including its source, phenotype and genotype.

The complete nucleotide sequence of the plasmid DNA should be provided. In addition, the identity, source, isolation and sequence of the gene encoding the antigen(s); a description of the steps involved in the construction of the entire plasmid; a detailed functional map of the plasmid; information on the source and function of component parts of the plasmid known to have biological activities, such as origins of replication, viral/eukaryotic promoters and other expression signals and genes encoding selection markers, should be provided. A clear rationale should be provided for the use of specific regions of DNA, such as the promoter or a gene encoding a selection marker and special attention should be given to the nature of a selection marker.
Any modifications to the original native sequence(s) of the antigen should be described and explained. The location of mammalian promoters in relation to antibiotic resistance genes and the use of novel promoters or inducers should be carefully considered. Certain sequences with properties of mobile elements, such as insertion sequences or retroviral-like long terminal repeats (LTRs), should be avoided. Oncogenes are not recommended unless justified. It is also recommended that genes encoding enzymatic activity or a biological function be either inactivated by genetic manipulation to remove any undesirable activity, or justified. Further, although the relevance at this stage may not be understood, as part of characterization, a DNA sequence homology check of the plasmid with the international databases (e.g. the National Center for Biotechnology Information, National Institute for Health, USA, and/or other international nucleotide databases) should be performed to investigate the presence of unintended sequences of biological significance such as those encoding cellular growth functions or alternative and unanticipated reading frames.

The identity of the plasmid after transfection into the bacterial cell to be used for production should be confirmed in addition to the phenotype of the cell. Representative restriction enzyme maps may be useful. Rearrangements of the plasmid within the host bacterial cell are not acceptable.

5.2.2.3 Cell banking system, characterization and testing

The production of a plasmid DNA vaccine should be based on a cell bank system involving an MCB and preferably a WCB. For early stage clinical trials it may be appropriate to use the MCB although sponsors are encouraged to prepare a WCB for later clinical studies.

A well-characterized bacterial cell containing the plasmid should be cloned and used to establish the MCB. The preparation of the MCB and WCB should be conducted according to GMP with appropriate precautions taken to prevent contamination. Information should be provided on the origin, form and storage conditions. Evidence for the viability of the MCB and WCB under storage and recovery conditions should also be provided by the time of application for marketing authorization. New WCBs should be fully characterized and meet established acceptance criteria. Specific phenotypic features that can form a basis for identification of the transformed cell should be described.

The DNA sequence of the entire plasmid should normally be confirmed at the stage of the MCB. Evidence that the MCB and WCB are free from extraneous microbial agents should be provided.

The genetic stability of the plasmid should be confirmed by characterization of the plasmid (copy number, size and sequence) after extended cell growth (end of production) at some stage during development.
5.2.3 **Process development and in-process control**

The developmental history of the manufacturing process should be provided. Tests and acceptance criteria for critical steps of the manufacturing process should be developed to ensure, and provide feedback on, the control of the process.

Validation of the manufacturing process should demonstrate reproducible and consistent clearance of process and host-related contaminants to levels acceptable for intended use in humans. Data from validation studies on the purification procedures may be required to demonstrate clearance of undesirable contaminants at each purification step and overall.

Process validation is not generally required for a product used in early-stage clinical trials although critical steps such as aseptic processing, sterilization of final product and cleaning validation—particularly when multi-product facilities or contract manufacturing organizations are used for the manufacturing—should be validated or carefully controlled prior to initiation of clinical development.

5.3 **Characterization**

5.3.1 **Characterization of bulk purified plasmid**

A summary of the characterization of the drug substance should be provided including its identity, strength, biological activity and purity. Rigorous characterization by chemical, physical and biological methods will be essential paying particular attention to the use of a wide range of analytical techniques which are based on different principles.

During development, the sequence of the entire plasmid should be determined. Attention should be paid to possible modification of the DNA because of the possibility that such modifications may influence the biological and immunological properties of the plasmid vaccine.

Potential impurities in the purified product should be described and investigated. These impurities include host cell residues, residual RNA and chromosomal DNA, materials used in the manufacturing process and media components. Data should be provided on the contaminants present in the purified plasmid, with estimates of their maximum acceptable or achievable levels. Denatured plasmid DNA and partial degradation by nucleases are typically observed as part of analytical procedures such as polyacrylamide gel electrophoresis, high performance liquid chromatography and capillary electrophoresis.

5.3.2 **Consistency of manufacturing**

A number of batches should be characterized as fully as possible to determine consistency. Any differences between one batch and another outside the accepted range for the parameters tested should be noted. The data obtained
from such studies should be used as the basis for the specification. During early clinical development, demonstration of consistency may be limited and occur as manufacturing experience is gained during the clinical development phases. Characterization of consistency of lots is generally done either during phase 3 or, if the phase 3 manufacturing process has not been scaled up for commercial manufacture, after phase 3 and prior to submission of a licence or marketing application.

5.4 Control of bulk purified plasmid

A specification for the drug substance should be established and justified. Descriptions of analytical methods and acceptance limits for the drug substance, including assay validation information should be provided. A summary of the results of testing of all batches produced should be provided.

It is recommended that the specification includes an assessment of the identity, nature and quantity of the plasmid, purity, biological activity, endotoxin content and sterility or bioburden. A justification of the specification should be provided. Early in development the specification may be limited and have wide acceptance criteria.

Not all the tests conducted during product characterization need to be carried out on each batch of vaccine. Some tests are required only to establish the validity or acceptability of a procedure, whereas others might be performed on a limited series of batches to establish consistency of production. Thus, a comprehensive analysis of the initial production batches should be undertaken to establish consistency with regard to identity, purity, potency and stability but thereafter a limited series of tests may be appropriate.

5.4.1 Identity

Each batch should undergo an appropriate selection of the tests used to characterize the purified plasmid in order to confirm its identity. However, the specific tests that adequately characterize any particular plasmid on a lot-to-lot basis, may depend on both the nature of the plasmid and its method of production and purification. Typically, restriction analysis will be the primary approach used to confirm identity; however, in vitro or in vivo expression of the plasmid accompanied by confirmation of the identity of the expressed antigen should also be considered.

5.4.2 Nature and quantification of plasmid, and biological activity

Quantification of the plasmid is usually by absorbance at 260 nm. The proportion of supercoiled plasmid should be determined and specifications set.
The biological activity of each batch of the drug substance should be determined using a suitable well-characterized assay together with an appropriate in-house reference preparation. For biological activity, whenever possible, the antigen should be expressed in vitro by transfection of a suitable cell line and the expressed protein characterized, for example, by immunofluorescence or by Western blot. Where possible, the in vitro assay should be shown to correlate with immunogenic activity or efficacy in an animal model. Alternatively, the plasmid should be shown to possess the relevant immunogenic or biological activity in an animal model.

5.4.3 **Purity**

Limits based on process capability and regulatory guidance should be established for all impurities detected and these should be identified and characterized as appropriate. The degree of contamination with chromosomal DNA, RNA and proteins should be assessed and limits established, and the criteria for rejection should be established and specified. It is important that the techniques used to demonstrate purity be based on as wide a range of physicochemical properties as possible. Where multiproduct facilities or contract manufacturing organizations are used for the manufacturing process, freedom from contamination with other products should be demonstrated.

5.5 **Reference standards or materials**

An in-house reference preparation should be established for use in assay standardization. Information on the reference standards or reference materials used for testing of the drug substance should be provided by the time of application for marketing authorization.

A suitable batch, preferably one that has been clinically evaluated, should be fully characterized in terms of its chemical composition, purity and biological activity, including, where possible, full sequencing, and retained for use as a chemical and biological reference material.

5.6 **Stability**

The stability assessment should be in compliance with the International Conference on Harmonisation (ICH) guideline Q5C “Stability testing of biotechnological/biological products”. The types of studies conducted, protocols used and the results of the studies should be summarized in an appropriate format such as tables, graphs or a narrative document. The summary should include results as well as drawing conclusions with respect to appropriate storage conditions and retest date or shelf-life. Limited stability information would be expected during initial clinical development. Further data on stability to support the expiry date of the drug substance for licence should be based on long-term, real-time stability studies under actual conditions of use.
6. **Manufacture and control of final formulated vaccine (drug product)**

6.1 **Composition**

The final composition of the vaccine should be documented.

6.2 **Manufacture**

A flow chart should be provided that illustrates the manufacturing steps from the bulk purified plasmid to the final formulated vaccine. The chart should include all steps (i.e. unit operations), identification of materials, major items of equipment and in-process controls. In some cases, this may involve simple dilution of the purified bulk; in other cases, a more complex formulation may be envisaged.

A description of each process step depicted in the flow chart should be provided. Information should be included on, for example, scale, buffers and other additives, major equipment, and process controls, including in-process tests and operational parameters with acceptance criteria.

6.3 **Control of materials**

Details of excipients, adjuvants or any other component of the vaccine in addition to the plasmid constituting the active substance should be provided, including their source, specification, method of conjugation, if appropriate, and final concentration in the vaccine.

6.4 **Control of final formulated vaccine**

A specification for the drug product should be established and justified. Descriptions of analytical methods and acceptance limits for the drug product, including information on assay validation should be provided. It is recommended that the specification includes an assessment of the identity, nature and quantity of the plasmid, purity, potency, endotoxin content and sterility. A justification of the specification should be provided. Early in development, the specification may be limited with wide acceptance criteria.

A summary of the results of the testing on all batches produced should be provided.

The appropriateness of performing tests on the bulk purified plasmid versus the formulated vaccine should be considered on a case-by-case basis and justified.

Several batches of vaccine, including the final dosage form, should be characterized as fully as possible to determine consistency. Any differences
between one batch and another should be noted. The data obtained from such studies should be used as the basis for the drug product specification.

Not all the tests conducted during product characterization need to be carried out on every batch of vaccine. Some tests are required only to establish the validity or acceptability of a procedure, whereas others might be performed on a limited series of batches to establish consistency of production. Thus, a comprehensive analysis of the initial production batches should be undertaken to establish consistency with regard to identity, purity, potency and stability but thereafter a more limited series of tests may be appropriate.

6.4.1 **Identity**

Each batch of vaccine should be subjected to an appropriate selection of the tests used to characterize the purified plasmid to confirm its identity. The specific tests that adequately characterize any particular plasmid on a lot-to-lot basis, however, may depend on both the nature of the plasmid and the method of its production and purification. Depending on the scope of identification tests, confirmation of the sequence or restriction enzyme mapping and verification of expression following transient transfection, will be necessary.

6.4.2 **Purity**

The purity of each batch of vaccine should be determined and be shown to be within specified limits. The analysis should include sensitive and reliable assays for contaminants of bacterial-cell origin and strict upper limits should be specified for their content in the bulk purified plasmid. Where multiproduct facilities or contract manufacturing organizations are used for the manufacturing process, freedom from contamination with other products should be demonstrated.

6.4.3 **Potency**

The potency of each batch of the vaccine should be determined using a suitable well-characterized assay together with an appropriate in-house reference preparation. A potency assay should be established that can be correlated to functional activity. This may take the form of a quantitative in vitro expression system or may require titration in a defined animal model to determine the minimum quantity of vaccine required to induce an appropriate immune response. The potency of the final vaccine formulation should be established unless otherwise justified and should be correlated with vaccine efficacy.

6.4.4 **Sterility**

Each batch of final product should be tested for sterility.
6.4.5 **Other tests**

The final product specification should include tests for endotoxin, visual appearance and pH. Other tests, such as the residual moisture, if the product is lyophilized, may be required to confirm the physical characteristics of the product as well as the formulation.

6.4.6 **Multi-component vaccines**

Additional factors must be considered when more than one plasmid forms the final formulated vaccine form. Plasmids in multi-component vaccines may encode additional antigens or cytokines or other biologically active molecules which enhance the efficacy or affect the safety of the vaccine. For each plasmid, the development overview, the control of production and the characterization of the bulk purified plasmid must be described as above. Careful consideration has to be given to the control of the final formulated vaccine. For example, potency may depend on the combination of plasmids and their interaction and not on any single plasmid component of a multicomponent vaccine.

6.5 **Reference materials**

A suitable batch of the final formulated vaccine, or bulk purified plasmid, preferably one that has been clinically evaluated, should be fully characterized in terms of its chemical composition, purity and biological activity, including, where possible, full sequencing, and retained for use as a chemical and biological reference material. This material should be used as the basis for evaluation of product quality for production batches.

6.6 **Stability**

Adequate stability studies form an essential part of vaccine development. The stability of the final product in the container proposed for use should, therefore, be determined and the results used to set a shelf life under appropriate storage conditions. Real time stability studies should be undertaken for this purpose, but accelerated stability studies at elevated temperatures may provide complementary supporting evidence for the stability of the product and confirm the stability indicating nature of the assays used to determine stability. The stability assessment should comply with ICH guideline Q5C.

7. **Nonclinical safety evaluation**

Although the recommendations set out below should be considered generally applicable, individual products may present particular safety concerns. The nonclinical safety evaluation should be considered on a product-specific basis taking into account the intended clinical use of the product. It is
important to note that when addressing some of the nonclinical and clinical issues a clear understanding of the product characteristics would be required to enable appropriate studies relating to the toxicology and pharmacology of the product to be designed.

7.1 Introduction

With the advent of DNA vaccines in the 1990s, several potential safety issues associated with administering plasmid DNA to humans were discussed: e.g. integration into the host’s chromosomes, immunopathological reactions, risks related to expression of cytokines or co-stimulatory molecules, antibodies against the injected DNA and potential biological activity of the expressed antigen. This revision of the WHO guidelines takes into account the accumulated experience of the use of DNA vaccines in both nonclinical and clinical development. Many phase 1 clinical trials in humans have been conducted with prophylactic DNA vaccines and at least one phase 2 clinical trial with a therapeutic vaccine is in progress. Studies using different doses of up to multi-milligrams, different schedules, routes of administration and delivery devices have been performed. In general, plasmid DNA appears to be safe and well-tolerated, i.e. no serious adverse reactions or major concerns related to the monitored parameters have been noted. At present, prime–boost strategies with other types of vectors, e.g. plasmid DNA/adenovirus and multiple immunization strategies are being investigated to improve immunogenicity. Many animal studies have been performed both with laboratory animals and with veterinary target animal species. It may be useful for sponsors to consult the published literature on experience with relevant animal models in the veterinary field.

7.2 General considerations on safety

This guidance is intended to address the issues specifically related to DNA vaccines. It should be read in conjunction with the guidance provided in the WHO guidelines on nonclinical evaluation of vaccines (2) and the nonclinical section (part A) of the WHO guidelines on clinical evaluation of vaccines: regulatory expectations (3).

The general aim of nonclinical safety evaluation is to determine whether new medicinal products have the potential to cause unexpected and/or undesirable effects. The study design should be based on the intended clinical use and focus on tests that are relevant for a plasmid DNA product. Classical safety or toxicological testing, as recommended for chemical drugs, will have limited application to a plasmid DNA product. As for other biotechnology products, studies with animal models for safety assessment consider two issues: 1) selection of the animal species and physiological state; and 2) the manner of delivery, including the dose, the route of administration and the treatment
regimen (frequency and duration). The nonclinical safety evaluation should provide information about a safe starting dose for the initial human trials. Using animal models to gain knowledge about the response to be expected in humans, always carries the risk that the animal species used in the model does not predict the human response or shows a non-relevant species-specific response. Relevant animal models should be used where possible, i.e. species/models whose immunogenic or biological response to the delivery system and expressed gene product would be expected to be similar to the response in humans.

The risk–benefit evaluation of a product is related to the actual product and its intended use. For example, a prophylactic DNA vaccine for use in healthy children will have a risk–benefit ratio different to that of a therapeutic vaccine against cancer for which there is no other available treatment. Thus, for these and other reasons, it is likely that a flexible approach will be necessary for the nonclinical safety evaluation of DNA vaccines.

If modifications to the manufacturing process or the DNA product are made during the development programme, the potential impact on the product should be considered. Modifications of the genetic sequence, the use of alternative promoter or enhancer sequences, or other changes to the product, may require additional nonclinical safety evaluation. The scientific rationale for the approach taken should be provided.

Although safety testing will undoubtedly be required, the range of tests that need to be carried out should be decided on a case-by-case basis, preferably in consultation with the national control authority. A suitable range of biological, molecular, biochemical, immunological, toxicological and histopathological investigative techniques should be used in the assessment of a DNA vaccine’s effect, over an appropriate range of doses during both acute and repeated exposure. Where there is experience with nonclinical and clinical use of a particular delivery vector by a particular route of administration, it may be possible to use information from previous studies or literature in place of further experimental work.

Several potential safety issues associated with administering plasmid DNA into humans are discussed below:

- The injected DNA taken up by cells of the host may integrate into the host's chromosomes and cause an insertional mutagenic event.
- The long-term expression of a foreign antigen may result in an undesired immunopathological reaction.
- The use of genes encoding cytokines or co-stimulatory molecules may pose additional risks.
- Antibodies may be formed against the injected DNA itself and these may contribute towards undesired autoimmune reactions.
• The expressed antigen may itself have biological activity.
• Expression of other gene sequences in mammalian or bacterial cells may pose a risk.

**DNA insertion**

It is known that DNA taken up by mammalian cells in culture can integrate into the cellular genetic material and be faithfully maintained during replication. This is the basis of the production of some recombinant therapeutic proteins. Theoretically, the introduction of extraneous DNA into a susceptible cell type in vivo could cause a transformation event leading to the formation of tumour cells by the insertion of an active oncogene, by insertional activation of a host-cell proto-oncogene or by insertional deactivation of a suppressor gene. DNA insertion can occur in one of three ways: by random integration of the plasmid into the genome, by homologous recombination between homologous sequences carried by the plasmid and the genome or by a direct integrating mechanism such as is employed by retroviruses. The most likely means in the present context would be random integration.

After injection of naked DNA into an animal, only a small proportion of the DNA molecules enter cells and of those only a fraction are likely to enter the nucleus. The probability of any DNA molecule integrating into a chromosome is very low. When consideration is given to the probability of insertional mutation occurring at a growth regulatory gene and to the multi-step process of oncogenesis, the risk of a tumorigenic event becomes exceedingly low.

Several investigators have assessed the ability of particular plasmids to integrate in vivo based on the association of plasmid DNA with genomic DNA after gel purification (6, 7). In most cases negative results have been obtained at a sensitivity in the region of one potential plasmid integration event per microgram of host-cell DNA (corresponding to approximately 150 000 diploid cells). However, alternative formulations or administration devices such as co-inoculation of a plasmid encoding a growth promoting factor, or electrostimulation, can lead to an increased potential for integration of plasmid DNA, and an investigation of the potential integration of the plasmid DNA in vivo into the host’s chromosomes should form part of the nonclinical safety testing of a DNA vaccine. Integration studies may not be necessary for a plasmid DNA vaccine if prior information on a similar plasmid, with the same mode of administration already exists. There would be a need to reassess integration if there was a significant change in the method of delivery, especially any change potentially involving an increase in the capacity of plasmid DNA to enter the nucleus.

**Immunopathological reaction**

Clinical studies to date have shown that plasmid DNA is well tolerated. Immunopathological reactions such as general immunosuppression and
inflammation have not been reported. Advances in understanding the mechanism of the immune response to an antigen which is expressed from injected DNA have been made although much remains unclear. Knowledge of the duration of expression of an antigen from injected DNA is limited although some reports suggest that expression could continue for many months, which means that the possibility of tolerance may remain a concern. In nonclinical investigations to date, tolerance has not been observed in adult animals and humans and the initial concern may have been overstated. Tolerance can be induced in neonatal mice; this may be because the mouse immune system at birth is immature and if development of tolerance is a concern for a specific product, a more relevant animal model is desirable.

Risks of genes encoding cytokines or co-stimulatory molecules

The co-administration of genes encoding regulatory cytokines or other immunostimulatory molecules has been used to improve the immune response to a DNA vaccine. However, such molecular adjuvants may have additional unintended consequences such as the possibility of stimulating one arm of the immune response at the expense of the other or, in theory, leading to immunopathological reactions, e.g. immunosuppression, chronic inflammation or autoimmunity. Considerable data on the safety (and usefulness) of this approach has accrued especially on their use in clinical trials of gene therapy and therapeutic studies with recombinant protein in humans; however, this approach should be followed carefully. Studies should continue to address the possibility that some cytokines may produce local or systemic toxic effects. The persistence of a cytokine expressing plasmid should be monitored. It cannot be ruled out that studies in animals responsive to the encoded human cytokine or models using the analogous animal genes may be useful to clarify safety issues.

Autoimmune reactions

Bacterial DNA can promote the production of IgG anti-DNA autoantibodies generally associated with the development of auto-immune glomerulonephritis in diseases such as systemic lupus erythematosus and this initially raised the theoretical concerns that DNA vaccination using bacterial plasmid DNA might induce such disease. Consistent with such a possibility, sensitive enzyme-linked immunosorbent assay analysis of serum samples from humans and animal models have shown that repeated DNA vaccination can stimulate a ≤ 5-fold increase in anti-DNA auto-antibody levels. Such an increase may not be detected by less sensitive clinical antinuclear antibody screening and the levels observed are well below that associated with the development of autoimmune disease. Although the possibility of anti-DNA antibody production should be considered in the development of plasmid DNA vaccines, analysis in the nonclinical programme is not generally warranted. Improvements in the efficiency of
DNA delivery and/or increases in vaccine dose and frequency may change the need for analysis of anti-DNA antibodies.

**Unwanted biological activity**

Consideration must be given to the possibility that the in vivo synthesized antigen may exhibit unwanted biological activity. If necessary, appropriate steps must be taken, e.g. by deletion mutagenesis, to eliminate this activity while retaining the desired immune response.

**Expression of other gene sequences**

If other gene constructs are included in the plasmid, such as antibiotic resistance genes for manufacturing reasons, then the possibility of expression of such gene sequences in mammalian cells or in microorganisms which are potentially pathogenic, and the possible clinical consequences of such expression, should be considered.

It is encouraging that data acquired to date demonstrate the safety of plasmid DNA. The data set is expanding, but the above issues must continue to be addressed, especially as measures are being sought to increase the efficacy of DNA vaccines.

### 7.3 Considerations on the nonclinical safety programme

In designing the nonclinical safety programme for a DNA vaccine product, the WHO guidelines on nonclinical evaluation of vaccines should be consulted in addition to the guidance provided here. Sponsors are also advised to perform a literature search for relevant published literature which should be used as part of safety evaluation and for justification of their safety programme.

Every product should be evaluated on a case-by-case basis. As a general rule, nonclinical safety assessment should be performed on every novel vaccine or vaccine/adjuvant formulation.

The following parameters should be considered and incorporated into the design of the nonclinical study: the nature of the vaccine, administration route (e.g. intranasal, intravenous, intramuscular or oral), formulation (e.g. liposome encapsulation, poloxamers) and any technique used to improve the uptake of the plasmid (e.g. electroporation). Data from a similar construct may be used to support the nonclinical safety assessment, but will require careful consideration.

When the drug product consists of more than one individual plasmid, testing of the drug product (formulated vaccine) is preferred unless there is reason to suspect one particular plasmid may present a significantly higher risk. The data from a drug product with several plasmids may be applicable for a drug product that contains a subset of the plasmids, unless there is interference between plasmids.
7.4 **Toxicity studies**

Studies should follow good laboratory practice (GLP) regulations and should test the final formulation intended for clinical use. However, certain assays might be conducted following the principles of GLP when newly developed methods have not been fully established.

The toxicity study may be combined with an assessment of local tolerance, immunogenicity and biodistribution evaluations. When possible, the dose, route and schedule should follow that intended for clinical use and the number of doses should be equal to or exceed that intended in the clinic. The dose interval can be shortened to 2–4 weeks or to another appropriate interval related to the intended dose regimen, although the kinetics of the immune response and potential toxic effect of the gene product should be considered. Relevant biodistribution parameters may also be investigated to allow any findings to be correlated with presence or expression of the gene product.

The animal model should be relevant and the product should be immunogenic in the chosen species. It is recommended that the study include an appropriate number of different doses and a vehicle or other appropriate control group. Toxicity assessments should be performed after both an acute and a recovery (follow-up) period, e.g. at 2–3 days and 14–21 days after the last administration. In general, studies in non-human primates are not required before proceeding to human trials. However, if the expected toxicity is species-specific, primates or transgene mouse models may be more predictive of clinical toxicity. Where possible the same lot of material should be used in the nonclinical safety evaluation and the initial clinical study.

Assessments should include daily clinical observations and injection site reactions, food consumption and body weight. Laboratory assessments should include clinical chemistry and haematology. Postmortem investigations should include macroscopic and microscopic assessments in an appropriate range of tissues, e.g. the injection site, spleen, liver, kidney, intestines, brain, bone marrow, ovaries/testes, lungs, lymph nodes, heart and adrenals. Findings should be assessed in relation to the pattern and severity of the effects and the relevance in relation to the intended product.

The studies should address product-specific concerns including the need for auto-antibody testing. In general, testing for anti-DNA-antibodies is no longer required. Local inflammatory response (e.g. myositis), organ-specific autoimmunity, immunopathology and other relevant parameters may need to be included. In particular where the encoded antigen is a self-antigen, or may show self-antigen mimicry, a wider range of studies, including auto-antibodies, may be necessary.
7.5 **Immunogenicity studies**

The purposes of the immunogenicity analysis are to define an appropriate dose, route, schedule and formulation of the vaccine for clinical trials and to provide justification (i.e. the benefit side of the risk–benefit equation) and rationale for the clinical trial. The results of assays to show immunological activity in an animal model should be presented. These could comprise antigen-specific antibody titres, serum neutralization titres, seroconversion rates, activation of cytokine-secreting cells and/or measures of cell-mediated immune responses. The studies should optimally be designed to give information about the duration of the immune response and may be combined with challenge or protection studies. If the DNA vaccine is intended to express, for example, a human cytokine, considerations should be given to the species-specificity, i.e. the animal species should be responsive to the cytokine, if possible, or a model using the corresponding animal cytokine may be used.

7.6 **Biodistribution, persistence and integration**

After inoculation of plasmid DNA into an experimental animal system, assays to assess the distribution, duration and potential integration of the plasmid should be performed, together with an assessment of the germ line, unless otherwise justified.

Biodistribution and persistence studies are required, unless substantial experience has already been gained with an almost identical or similar product. Biodistribution and persistence should be investigated using, for example, sensitive nucleic acid detection techniques, and the justification for the chosen assays should be stated. The assay limit of sensitivity, its specificity, and the potential for tissue- or preparation-specific inhibitors should be established during the assay validation. Among the nucleic acid detection techniques, quantitative polymerase chain reaction (PCR)-based assays have been the most commonly used and most reliable means of assessing plasmid levels in biodistribution and integration studies. The amount of plasmid in the relevant tissues should be quantified and the persistence of plasmid at each site over time should be monitored at an appropriate number of time-points, both early (e.g. 1–7 days) and late (e.g. 2-3 months). The duration and sites of expression of the encoded proteins over time should be investigated. If the encoded protein product is expected to persist for a considerable length of time, the impact of this should be addressed.

Size fractionation/gel-purification assays, where plasmid co-migrates with genomic DNA, may be used as a parameter for potentially integrated plasmid DNA (6, 7). The sensitivity and specificity of the chosen assay should be well-documented.
Published studies on biodistribution of DNA vaccines indicate that intramuscular, subcutaneous, intradermal or particle-mediated delivery does not result in long-term persistence of plasmid at ectopic sites. Some studies have reported ≤ 30 copies of plasmid per 100,000 host cells persist at the site of injection, after 60 days (8–10), whereas other studies have shown that the plasmid can persist at the injection site at levels greater than 500 copies per 100,000 cells 6 months post-administration (7). If ≤ 30 copies of plasmid DNA per 100,000 host cells persist after 60 days, then further integration assessment may not be necessary. As more experience is gained, this issue will need to be addressed again.

Depending on the potential for integration and the proposed clinical indication, further studies may be required to investigate directly any actual integration, e.g. by specially designed PCR-primers (11), or the potential for tumour formation or disruption of normal gene expression.

It may also be necessary to investigate the distribution and clearance of the material used for delivery of the DNA, e.g. complexing material, if sufficient information is not already available in the published literature. The studies should identify sites of uptake after in vivo delivery.

7.7 Genotoxicity

The standard battery of genotoxicity and conventional carcinogenicity studies is not applicable to DNA vaccines. However, genotoxicity studies may be required to address a concern about a specific impurity or novel chemical component, e.g. a complexing material that has not been tested previously.

7.8 Developmental and reproductive toxicity

Integration into reproductive tissue may result in germline alteration. The possibility of distribution to, integration or expression in germline cells must be investigated unless otherwise justified, e.g. the clinical indication or patient population indicates that such studies are not warranted. Confirmation of absence of germline alterations may be gained from investigating the presence and persistence of the plasmid DNA in gonadal tissue from both male and female animals, using e.g. PCR technology. Persistence of plasmids in gonadal tissue over time require further investigation, e.g. of ova and sperm cells and considerations of potential effects on fertility and general reproductive function. In addition, embryo-fetal and perinatal toxicity studies may be required if women of childbearing potential are to be exposed to the product, depending on intended clinical use and population. Such studies may not be required prior to clinical studies in populations with life-threatening diseases, provided appropriate measures are taken to minimize risks. Before a DNA vaccine is used in children or newborns, it
should be tested for safety and immunogenicity in adults, and appropriate nonclinical models, e.g. with juvenile animals, should be considered for the study of toxicity and induction of immunological tolerance.

Authors

The draft of these revised Guidelines was prepared by Dr J. Robertson, National Institute for Biological Standards and Control, Potters Bar, England; Mr J. Ackland, The Biologics Consulting Group, San Mateo, California, USA; Dr A. Holm, Danish Medicines Agency, Copenhagen, Denmark.

Acknowledgements

Acknowledgements are due to the following experts for their comments, advice and information given at an Informal WHO Consultation on the WHO guidelines for assuring the quality and preclinical safety evaluation of DNA vaccines held at WHO Headquarters, Geneva, from 4–5 July 2005:

Dr T. Bektimirov, L.A. Tarasevich State Research Institute for Standardization and Control of Medical Biological Preparations, Moscow, Russian Federation; Dr K. Cichutek, Paul-Ehrlich-Institute, Langen, Germany; Mr D. Daout, Serum Institute of India, Ltd, Nyon, Switzerland; Dr F. Ferre, Althea Technologies Inc., San Diego, California, USA; Dr D. Gannaway, PowderMed Ltd, Oxford, England; Dr H. Golding, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Maryland, USA; Dr J. Joung, Korea Food and Drug Administration, Seoul, Republic of Korea; Dr D. Kaslow, Vical Incorporated, San Diego, California, USA; Dr A. Kojima, National Institute of Infectious Diseases, Tokyo, Japan; Dr R. Krause, International Federation of Pharmaceutical Manufacturers Associations, Geneva, Switzerland; Dr B. Ledwith, Merck Research Laboratories, West Point, Pennsylvania, USA; Dr P. Manyike, South African AIDS Vaccine Initiative, Cape Town, South Africa; Dr S. Phumiamorn, Department of Medical Sciences, Ministry of Public Health, Thailand; Dr T. Richie, Naval Medical Research Center, Silver Spring, Maryland, USA; Dr G. Sharpe, Cobra Bio-Manufacturing plc., Keele, Staffordshire, England; Dr G. Thiry, International AIDS Vaccine Initiative, New York, USA; Dr J. Ulmer, Chiron Corporation, Emeryville, California, USA; Dr J. van der Laan, National Institute for Public Health and the Environment, BA Bilthoven, The Netherlands; Dr Y. Wang, National Institute for the Control of Pharmaceutical and Biological Products, Temple of Heaven, Beijing, People’s Republic of China; Dr D. Weiner, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA.

Thanks are also due to the following for their written comments and advice:

Dr D. Gannaway, PowderMed Ltd., Oxford, England; Dr J. Joung, Korea Food and Drug Administration, Seoul, Republic of Korea; Dr D. Kaslow, Vical Incorporated, San Diego, California, USA; Dr J. Lebron, Merck Research Laboratories, West Point, Pennsylvania, USA; Dr S. Phumiamorn, Department of Medical Sciences, Ministry of Public Health, Thailand; Dr F. Reigel, Biological Medicines and Laboratories, Swissmedic Agency for Therapeutic Products, Berne, Switzerland; Dr R. Sheets,
Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA; Dr G. Thiry, International AIDS Vaccine Initiative, New York, USA; Dr G. Vicari, Via Principessa Clotilde, Rome, Italy.

WHO Secretariat


References


Annex 2

Recommendations for inactivated rabies vaccine for human use produced in cell substrates and embryonated eggs

These Recommendations provide information and guidance to national regulatory authorities and vaccine manufacturers concerning the characterization, production and control of rabies vaccines in order to facilitate their international licensure and use. Each of the following sections constitutes a recommendation. The parts of each section that are printed in normal type have been written in the form of requirements so that if a national regulatory authority so desires these parts may be adopted as definitive national requirements. The parts of each section printed in small type are comments and additional guidance. It is recommended that modifications to these Recommendations be made only on condition that the modifications ensure that the vaccine is at least as safe and efficacious as that prepared in accordance with the Recommendations set out below.

In order to facilitate the international distribution of vaccine made in accordance with these recommendations, a summary protocol is given in the Appendix.

Introduction
General considerations
Part A. Manufacturing recommendations
  A.1 Definitions
  A.2 General manufacturing recommendations
  A.3 Control of source materials
  A.4 Control of vaccine production
  A.5 Filling and containers
  A.6 Control tests on final product
  A.7 Records
  A.8 Samples
  A.9 Labelling
  A.10 Distribution and shipping
  A.11 Stability, storage and expiry date
  A.12 Intradermal route of administration
Part B. Nonclinical evaluation of rabies vaccines
Part C. Clinical evaluation of rabies vaccines
Part D. National control requirements
  D.1 General
  D.2 Release and certification

Authors
Acknowledgements
References
Appendix
Summary protocol
Introduction

The last revision of the requirements for rabies vaccines for human use was in 1980 (1). However, an additional document, WHO Requirements for rabies vaccine (inactivated) for human use produced in continuous cell lines was published in 1987 to take into consideration advances in the development of cell culture derived vaccines (2). An amendment which updated the section on the International Standard for Rabies Vaccine was published in 1994 (3, 4).

The following Recommendations are for inactivated rabies vaccine for human use produced only in cell substrates and embryonated eggs. They replace all previous requirements (1–4). The scope of the present recommendations encompasses vaccines produced in cell substrates, ranging from primary cells (hamster kidney and chick embryo fibroblasts), diploid cells, to continuous cell lines such as Vero cells. Purified vaccines produced using duck embryos are also within the scope of the document.

However, vaccines produced in mammalian neural tissues are not considered in this or any other document because their use is no longer recommended.

General considerations

Rabies is an under-reported, neglected and deadly disease estimated to cause more than 50,000 human deaths annually, most of which occur in the poorest regions of the world (5). Once clinical symptoms are evident, the prognosis for survival is poor and death is almost inevitable. The population at risk includes 2.5 billion people currently living in regions in which rabies is endemic. Half of the victims of dog bites and rabies subsequent deaths from occur in children younger than 15 years of age, as they are the population most at risk. (6). Clearly, greater efforts should be made to improve the control of rabies, a zoonotic disease with the highest case fatality rate known to humans.

One of the most important elements in the effective control of human rabies is the use of efficacious vaccines. Vaccines produced in mammalian neural tissues have been in use for more than 100 years. However, it is the availability and use of rabies vaccines produced in cell culture and embryonated eggs that has dramatically decreased the number of human deaths throughout the world, most notably in countries where canine rabies is endemic. For example, in Thailand, the introduction of cell culture vaccines together with reduced dosage intradermal regimens decreased the incidence of human rabies by 80% in 15 years (5). Similar progress has been documented in other countries where nerve tissue vaccines have been replaced by rabies vaccines produced in cell culture and embryonated eggs.
This document focuses on the recommendations for production, control and evaluation of rabies vaccines, which as stated above, are one of the most important elements in the effective control of human rabies. However, vaccine should always be considered as part of the complete treatment and additional information on recommendations for the treatment of the disease is available in the Report of the Expert Consultation on Rabies (7).

WHO requirements for rabies vaccines were published in 1981 and 1987. The former requirements encompassed vaccines derived from mammalian neural tissue as well as vaccines produced using embryonated eggs and variety of cell substrates, whereas the latter covered only vaccines produced in continuous cell lines. Since that time, there have been many developments in the production and quality control of vaccines as well as in their overall regulation. In particular, considerable attention has been given to safety issues.

The scientific basis for the present revision of the requirements for rabies vaccines was developed at the meetings of a working group held at WHO, Geneva, in May 2003 and May 2004. The issues identified for revision were: the scope of the document; the substrates for vaccine production that the revised document would cover; the inactivation process; the test for effective inactivation; potency test; the use of in vitro assays for determination of the antigen content as a measure of consistency of production; stability test and the value of the accelerated degradation test; and national regulatory authority requirements. Further details of these discussions and the rationale for the proposed revisions are available in the meeting reports (8, 9).

Rabies vaccines produced in mammalian neural tissues (brain of adult animals such as sheep and goats; brain of suckling animals such as mouse, rat and rabbit) have been in use worldwide for many years. It is well known that their use has led to adverse reactions following immunization, such as encephalomyelitis and polyneuritis (10, 11). Although the risk of such adverse reactions is reduced when the virus is grown in the brains of newborn animals, such as rats and mice, before the development of myelin in the brain, the safety profile of these vaccines is still unacceptable. Moreover, there is evidence for a lack of potency of these neural tissue vaccines, leading to inadequate protection in humans, making a strong argument for the discontinuation of their production and use (12–14). The present revised recommendations are intended to improve control of rabies disease by promoting vaccines of assured quality as part of pre-exposure vaccination and post-exposure prophylaxis. To facilitate the international distribution of vaccine produced in accordance with these recommendations, a summary protocol is given in the Appendix.

Recently developed methods for genetic sequencing of rabies virus have been considered in this document. Given that the genetic characteristics are
part of the identity of the vaccine strains, it would be beneficial to include
this information in the licensing of new vaccines and to use sequencing in
monitoring for subtle genetic changes of vaccine strains over time.

The approach to potency testing remains the same as previously recommended.
The National Institute of Health (NIH) potency test, based on a mouse
protection assay, is recognized as a reliable assay. A review of the data on a
single-dilution NIH test has led to the development of criteria for the validation
of a modified NIH assay. The latter has been extensively used for lot release of
rabies vaccine for veterinary use while the experience in testing vaccines for
human use is still at the experimental stage. More data are needed to support
this approach and to provide a basis for a standardized testing procedure.

Several studies conducted over the last 10 years have provided useful
information on the value and potential use of the in vitro assays for
measurement of the antigen content in vaccines (National Institute for
Biological Standards and Control (NIBSC) and Agence Française de Sécurité
Sanitaire des Produits de Santé (AFSSAPS) studies). Such assays have been
successfully used by several manufacturers to control antigen concentration
during production and in the final formulation of a product. However, in
vitro data concerning antigen concentration in the final vaccine have not
generally been reported and direct correlations between such determinations
and evidence of protection in humans need to be evaluated. Correlations
between the in vitro assays and the NIH test have proven challenging owing
primarily to the variability of the NIH test. Further characterization of the
reagents used for in vitro assays may clarify the potential of these methods
to assess the quality as well as the quantity of antigen. Both manufacturers
and national regulatory authority/national control laboratory staff are
encouraged to further develop and use these assays, and to accumulate more
data on their application to the control of rabies vaccines.

Because the intradermal route of administration has been used for some
rabies vaccines initially developed for intramuscular administration, some
additional considerations are discussed in a separate section of this document
(see Intradermal route of administration). Furthermore, guidance for
nonclinical and clinical testing of vaccines intended to be administered by
the intradermal route are provided in sections B and C of this document.

Given that new rabies vaccines might be developed in the near future, a section
on clinical evaluation of vaccines describes specific considerations for the
evaluation of data generated in clinical trials and provides some guidance on
how to assess the immunogenicity and safety of rabies vaccines.

The stability evaluation of vaccines is addressed in a separate section of
these recommendations. The importance of real-time studies under intended
storage conditions is emphasized.
Relevant guidance documents published since the last revision of the requirements for rabies vaccines have been considered in the present revision. The following documents are mentioned as relevant and should be consulted for further information. Updated requirements for the characterization of continuous cell lines used for the preparation of biologicals, adopted in 1998 (15), provide current recommendations for vaccines produced using cell substrates. It is important to note that the WHO recommendation for 10 ng of residual host cell DNA per single human dose for products manufactured using continuous cell lines remains the same as recommended in 1996. In addition, guidance to reduce possible risk of contamination of vaccines by transmissible spongiform encephalopathies (16) and updated requirements for blood products (17) including human albumin, used in some vaccines as a stabilizer, are now available.

In recent years concerns have been raised over the safety of thiomersal in vaccines, especially those given to infants. These concerns have been based primarily on data regarding the toxicity of a related substance, methyl mercury, and from data on chronic exposure to mercury via the food chain. Such safety concerns have led to initiatives in some countries to eliminate, reduce or replace thiomersal in vaccines, both in monodose and multidose preparations. It is important to note that concerns about the toxicity of thiomersal are theoretical and there is currently no compelling scientific evidence of a safety problem with its use in vaccines, although public perception of risk remains in some countries. WHO policy is clear on this issue, and the Organization continues to recommend the use of vaccines containing thiomersal for global immunization programmes because the benefits of using such products far outweigh any theoretical risk of toxicity (18).

Part A. Manufacturing recommendations

A.1. Definitions

A.1.1 International name and proper name

The international name should be rabies vaccine for human use. The proper name should be the equivalent name in the language of the country of origin.

The use of the international name should be limited to vaccines that satisfy the requirements formulated below.

A.1.2 Descriptive definition

Rabies vaccine for human use is a freeze-dried or liquid preparation of well characterized, laboratory adapted and attenuated virus with stable biological characteristics, grown in cell substrates or embryonated eggs, and inactivated
by a suitable method. The preparations for human use should satisfy all the recommendations formulated below.

A.1.3 **International standards**

The fifth International Standard for Rabies Vaccines was established by the WHO Expert Committee on Biological Standardization in 1991, with a potency of 16 IU of Rabies Vaccine per ampoule. Recent research has indicated that the glycoprotein and ribonucleoprotein components of inactivated rabies vaccines play an important role in conferring protection. For this reason, the Committee also assigned 10 IU of Rabies Virus PM-Glycoprotein and 135 IU of Rabies PM-Ribonucleoprotein to the contents of each ampoule of the International Standard. It is recognized, however, that these components might differ antigenically in the different virus strains used for vaccine production; the International Standard may therefore be inappropriate for the estimation of glycoprotein and ribonucleoprotein components of vaccines not derived from the Pitman-Moore (PM) strain.

The Second International Standard for Rabies Immunoglobulin was established by WHO in 1993. It is a preparation of human immunoglobulin and each ampoule contains 30 IU.

The fifth International Standard for Rabies Vaccine and the first International Standard for Rabies Immunoglobulin were initially held at the Statens Serum Institute in Copenhagen, Denmark. Since 1997 this standard has been in the custody of the National Institute for Biological Standards and Control, Potters Bar, Hertfordshire, EN6 3QG, England (web site: http://www.nibsc.ac.uk).

The international reference materials mentioned above are intended for the calibration of national reference materials for use in the quality control of rabies vaccines. They are distributed free of charge, on request, to national control laboratories. The WHO catalogue of international biological standards should be consulted for the latest list of appropriate international standards and reference materials (http://www.who.int/biologicals/IBRP/Catalogue.htm).

A.1.4 **Terminology**

The following definitions are given for the purpose of these Recommendations only.

*Adventitious agents:* contaminating microorganisms including bacteria, fungi, mycoplasmas, and endogenous and exogenous viruses that have been unintentionally introduced.

*Cell seed:* a quantity of well-characterized cells of human or animal origin stored frozen at −100 °C or below 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.

**Final bulk:** The material after completion of preparations for filling, homogenous with respect to mixing of all components, and present in the container from which the final containers are filled. The final bulk may be prepared from one or more purified bulk materials.

**Final lot:** a collection of sealed final containers of freeze-dried or liquid vaccine that is homogeneous with respect to the risk of contamination during the filling process or the preparation of the finished vaccine. A final lot must therefore have been filled or prepared in one working session from a single final bulk.

**Master cell bank:** a quantity of fully characterized cells of human or animal origin stored frozen in liquid nitrogen in aliquots of uniform composition derived from the cell seed, one or more of which may be used for the production of a manufacturer’s working cell bank.

**Production cell culture:** a collection of cell cultures 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.

**Purified bulk material:** a pool of inactivated and processed single harvests before preparation of the final bulk. The pool may be prepared from one or more single harvests and may yield one or more final bulks.

**Single virus harvest:** a virus suspension of the same virus working seed lot inoculated, incubated and harvested together from either a group of embryonated eggs or a cell culture in one production run. Multiple harvests from the same production cell culture may be pooled and considered a single virus harvest.

**Virus master seed lot:** a quantity of virus, physically homogeneous, that has been prepared as a single lot. It is used for the preparation of working seed lots.

**Virus working seed lot:** a seed lot prepared from the master seed lot with no more than five passages removed from the master seed lot. Both passage level and the method of passaging should be approved by the national regulatory authority.

**Working cell bank (WCB):** a quantity of cells of uniform composition derived from one or more ampoules of the master cell bank, which may be used for the production cell culture. In normal practice, a cell bank is expanded by serial subculture up to passage number (or population doubling, as appropriate) selected by the manufacturer, at which point the cells are combined to give a single pool and preserved cryogenically to
form the WCB. One or more of the cryotubes from such a pool may be used for the production of cell culture.

A.2 General manufacturing recommendations

The general requirements for manufacturing establishments contained in Good manufacturing practices for pharmaceuticals (19) and Good manufacturing practices for biological products (20) should apply to establishments manufacturing inactivated rabies vaccine, with the addition of the following:

Rabies has the highest case-fatality rate of any currently recognized infectious disease; therefore the assignment of an appropriate biosafety level for specific work with the virus at the production as well as at the control facilities is an essential precautionary measure.

The assignment of a virus to a biosafety level for production and quality control facilities must be based on a risk assessment. Such an assessment will take the risk group, as well as other factors, into consideration in establishing the appropriate biosafety level. For example, a virus assigned to risk group 2 may generally require Biosafety Level 2 facilities, equipment, practices and procedures for safe conduct of work. However, if particular phases of production require work with live virus and/or exposure to large quantities and/or high titre of virus as well as exposure to aerosol, then Biosafety Level 3 may be more appropriate to provide the necessary degree of safety, because it ensures superior containment in the production and quality control facilities. The biosafety level assigned for the specific work is therefore a result of professional judgement based on a risk assessment rather than an automatic assignment of a laboratory biosafety level according to the particular risk group designation of the pathogenic agent to be used. Further guidance on the risk assessment and assignment of appropriate biosafety level are available in the WHO laboratory biosafety manual (21). However, countries should draw up their own national classification of microorganisms, by risk group.

Personnel employed in the production and control facilities should be healthy and should receive regular medical examinations. They should be adequately trained and protected against accidental infection with rabies virus. Steps should be taken to ensure that all the personnel in the production and control areas have been immunized against rabies and maintain an antibody titre of at least 0.5 IU per ml of serum as measured by the rabies fluorescent focus inhibition test (RFFIT) and fluorescent anti-virus neutralization (FAVN). Periodic titre control and if required, booster injections are recommended for people who are at continuous risk of rabies exposure. Further guidance on the need for boosters is available in the Report of the Expert Consultation on Rabies (7).
• Taking a full body shower upon exit from the BSL-3 enhanced (pandemic influenza vaccine) containment facility is recommended. It is mandatory following situations when staff may have been exposed to vaccine virus.
• Personnel should be instructed, in a written document to which they sign their agreement, not to have contact with animals, in particular farm animals 14 days following departure from the facility where vaccine has been produced. Currently the risks involved in contact with household dogs and cats are not considered to be significant, but the available scientific evidence is sparse.
• Staff should be prophylactically vaccinated with seasonal influenza vaccines.
• It is anticipated that before large-scale vaccine production is attempted, pilot lots of pandemic strain vaccine will have already been produced. Experimental vaccines inducing protective antibody levels are recommended for use by staff before large-scale vaccine production commences, if possible.
• Antiviral treatment must be available as necessary.

3.5 Biosafety management and implementation within a vaccine production facility

3.5.1 Management structure

The implementation of the biosafety levels described in these guidelines requires that the institution employ a biosafety officer who is knowledgeable in large-scale viral production and containment, but is independent of production in his or her reporting structure. The biosafety officer is responsible for the independent oversight of the implementation of the biosafety practices, policies and emergency procedures in place within the company or organization and should report directly to the highest management levels within the company. A biosafety officer is needed in addition to a qualified person who, in some countries, has overall responsibility for a medicinal product.

There should also be a Biosafety Committee comprising representatives of viral production and quality control that is responsible for reviewing the biosafety status within the company and for coordinating preventive and corrective measures. The institutional biosafety officer must be a member of the Committee. The chairperson should be independent of both the production and quality control functions. The management and governing board of the manufacturing company should ensure that adequate priority and resources are made available to the Committee to implement the required measures.

3.5.2 Medical surveillance

Occupational health departments at vaccine manufacturers of pandemic strain influenza vaccines should provide training in recognizing the clinical signs of influenza infection to company physicians, nurses and vaccine
manufacturing supervisors, who must make decisions on the health of personnel associated with the manufacture and testing of pandemic strain influenza vaccine. Local medical practitioners caring for personnel from the manufacturing site should receive special training in the diagnosis and management of pandemic influenza infection. Any manufacturer embarking on large-scale production should have documented procedures for dealing with influenza-like illness in the staff involved, or their family members, including diagnostic procedures and prescribed treatment protocols. Manufacturers should ensure that staff understand that they have an obligation to seek medical attention and to report any influenza-like illness to the occupational health department or equivalent. Manufacturers should hold supplies of one or more effective antiviral agent(s) and have defined means of quarantining staff if necessary.

3.5.3 Implementation

A detailed and comprehensive risk analysis should be conducted to define possible sources of contamination of personnel or the environment that may arise from the production or testing of live influenza virus within the establishment. For each procedure or system, this analysis should take into account the concentration and stability of the virus at the site, the potential for inhalation or injection that could result from accidents, and the potential consequences of a major or minor system failure. The procedural and technical measures to be taken to reduce the risk to workers and the environment should be considered as part of this analysis. The results of this risk analysis should be documented.

A comprehensive Biosafety Manual must be created and implemented that fully describes the biosafety aspects of the production process and of the quality control activities. It should define such items as emergency procedures, waste disposal, and the requirements for safety practices and procedures as identified in the risk analysis. The manual should be made available to all staff of the production and quality control units, with at least one copy present in the containment area(s). The manual should be reviewed and updated when changes occur and at least annually.

Comprehensive guidelines outlining the response to biosafety emergencies, spills and accidents should be prepared and made available to key personnel for information and for coordination with emergency response units. Rehersals of emergency response procedures are helpful. These guidelines should be reviewed and updated annually.

The implementation of the appropriate biosafety level status in the production and testing facilities should be verified through an independent assessment. National requirements concerning verification mechanisms should be in place and complied with.
<table>
<thead>
<tr>
<th>Vaccine virus</th>
<th>Haemagglutinin receptor specificity</th>
<th>Tests needed on reference virus&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Proposed containment for vaccine production</th>
</tr>
</thead>
<tbody>
<tr>
<td>H5, H7 reassortants, from HP viruses&lt;sup&gt;b&lt;/sup&gt;</td>
<td>α2,3 residues</td>
<td>Ferret, chicken, sequence, plaquing, egg embryo</td>
<td>BSL-2 Enhanced (pandemic influenza vaccine)</td>
</tr>
<tr>
<td>H5, H7 reassortants, from NP viruses&lt;sup&gt;b&lt;/sup&gt;</td>
<td>α2,3 residues</td>
<td>Ferret, chicken, sequence, plaquing, egg embryo</td>
<td>BSL-2 Enhanced (pandemic influenza vaccine)</td>
</tr>
<tr>
<td>Non-H5, H7 reassortant</td>
<td>α2,3 residues</td>
<td>Ferret</td>
<td>BSL-2 Enhanced (pandemic influenza vaccine)</td>
</tr>
<tr>
<td>Non-H5, H7 reassortant</td>
<td>α2,6 residues</td>
<td>Ferret</td>
<td>BSL-2 Enhanced (pandemic influenza vaccine)</td>
</tr>
<tr>
<td>H5, H7 HP viruses</td>
<td>α2,3 residues</td>
<td>Not applicable</td>
<td>BSL-3 Enhanced (pandemic influenza vaccine)</td>
</tr>
<tr>
<td>H5, H7 NP viruses</td>
<td>α2,3 residues</td>
<td>Ferret, chicken, sequence, plaquing, egg embryo</td>
<td>BSL-2 Enhanced (pandemic influenza vaccine)</td>
</tr>
<tr>
<td>Non-H5, H7 viruses</td>
<td>α2,3 residues</td>
<td>Ferret</td>
<td>BSL-2 Enhanced (pandemic influenza vaccine)</td>
</tr>
<tr>
<td>Non-H5, H7 viruses</td>
<td>α2,6 residues</td>
<td>Ferret</td>
<td>BSL-2 Enhanced (pandemic influenza vaccine)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Test performed by WHO reference laboratory.

<sup>b</sup> Highly pathogenic and nonpathogenic viruses.
Authors

Four background documents\(^1\) were discussed in a teleconference on 27 July 2005 convened by the World Health Organization, Geneva, Switzerland (Dr D. Wood, S. Lambert, A. Mohammadi and B. Kay) attended by the following persons: Dr P. Celis, European Medicines Agency, London, England; Mr T. Colegate, Chiron Vaccines, Liverpool, England; Dr J. Katz, Centers for Disease Control, Atlanta, USA; Dr C. Gerdil, Sanofi Pasteur, Marcy l’Etoile, France; Dr G. Grohmann, Therapeutic Goods Administration, Woden ACT, Australia; Dr A. Hampson, WHO Collaborative Centre for Influenza, Parkville, Victoria, Australia; Dr A. Hay, WHO Collaborative Centre for Influenza, National Institute for Medical Research, London, England; Dr R. Levandowski, Food and Drug Administration, Bethesda, Maryland, USA; Mr P. Logan, Health and Safety Executive, Merseyside, England; Dr J. Robertson, National Institute for Biological Standards and Control, Potters Bar, Herts, England; Dr D. Swayne, Department of Agriculture, USA; Mr J. Richmond, Atlanta, Georgia, USA.

A first draft document was prepared by the WHO Secretariat (Dr D. Wood) based on the outcome of the teleconference and the commissioned papers. Comments on this first draft were received from Dr Alexander, Dr A. Hampson, Dr A. Hay, Dr P. Logan, Dr J. Robertson, Dr D. Swayne and the International Federation of Pharmaceutical Manufacturers (IFPMA) Influenza Vaccine Supply International Task Force. A version of the document for public comment (WHO/BS/05.2026) was prepared by the WHO Secretariat (Dr D. Wood) taking into account the comments received and further review by Dr J. Robertson and Dr J. Wood.

The final draft version of the document (WHO/BS/05.2026, 12 October 2005) was prepared by the Secretariat (Dr D. Wood and Dr S. Lambert) taking into account comments from participants at a WHO informal consultation on WHO/BS/05.2026, held in Geneva from 19–20 September 2005 attended by the following persons: Mr T. Colegate, Chiron Vaccines, Liverpool, England; Dr G. Grohmann, Therapeutic Goods Administration, Woden ACT, Australia; Dr I. Kallings, Swedish Institute for Infectious Disease Control, Solna, Sweden; Dr T. Kurata, National Institute of Infectious Diseases, Tokyo, Japan; Dr Y. Lawanprasert, Food and Drug Administration, Nonthaburi, Thailand; Dr P. Logan, Merseyside, England; Dr J. Lubroth; Food and Agriculture Organization of the United Nations (FAO), Rome, Italy; Dr P. Payette, Public Health Agency of Canada, Ottawa, Canada; Mr S. Phoshoko, National Department of Health, Pretoria, South Africa; Dr I. Raw, Instituto Butantan, São Paolo, Brazil; Dr J. Richmond, Southport, North Carolina, USA; Dr J. Robertson, National Institute for Biological Standards and Control, Potters Bar, Herts., England; Dr J-F Saluzzo, Sanofi Pasteur, Marcy l’Etoile, France; Dr N.T. Van,

\(^1\) The following series of background papers, commissioned by the WHO Secretariat, were prepared in the period April–July 2005.

\(a\) A review of WHO biosafety guidelines for Manufacturing Avian Influenza Vaccines (Frey, Richmond, Robinson).

\(b\) A risk assessment for large scale manufacture of inactivated influenza vaccines from reassortants derived from avian influenza viruses (Wood, Robertson, Logan).

\(c\) Industry pandemic biosafety position paper (IFPMA influenza vaccine supply international task force).

\(d\) Conceptual risks of reassortants for the environment (Swayne).
Vabiotech, National Institute of Hygiene and Epidemiology, Hanoi, Viet Nam; Dr T.G. Webster, St. Jude’s Children’s Research Hospital, Memphis, Tennessee, USA; Dr J. Wood, National Institute for Biological Standards and Control, Potters Bar, Herts, England. The WHO Secretariat included Dr L. Chocarro, Access to Technologies; Dr B. Kay, Communicable Disease Surveillance and Response; Dr S. Lambert, Quality and Safety of Biologicals; Dr A. Mohammadi, Communicable Disease Surveillance and Response; Dr N. Previsani, Communicable Disease Surveillance and Response; Dr Y. Pervikov, Initiative for Vaccine Research; Dr J. Sokhey, WHO Regional Office for South-East Asia, New Delhi, India; Dr K. Stohr, Global Influenza Programme; Dr D. Wood, Quality and Safety of Biologicals and Dr W. Zhang, Global Influenza Programme.

Gratitude is also due to the following individuals for their written comments: Dr A. Hampson, WHO Collaborative Centre for Influenza, Parkville, Victoria, Australia; Dr I. Kallings, Swedish Institute for Infectious Disease Control, Solna, Sweden; Dr P. Payette, Public Health Agency of Canada, Ottawa, Canada; Dr J. Robertson, National Institute for Biological Standards and Control, Potters Bar, Herts., England; Dr J. Wood, National Institute for Biological Standards and Control, Potters Bar, Herts., England: and the Influenza Vaccine Supply Task Force.

References


Appendix 1

Testing for attenuation of influenza vaccine strains in mammals

Titration of test virus

The dose of vaccine virus or parental strain virus that produces infection in 50% of cases should be determined by titration in eggs (EID$_{50}$) or cell culture (TCID$_{50}$), as appropriate. Titration of vaccine virus stock and parental virus stocks should be determined within the same laboratory and titres should be sufficiently high that these viruses can be compared using equivalent high doses in mice or ferrets (10$^7$ to 10$^6$ EID$_{50}$ or TCID$_{50}$).

Ferrets

Experimental procedure

Outbred ferrets 4–8 months of age are sedated either by intramuscular inoculation of a mixture of anaesthetics (e.g. ketamine (25 mg/kg), xylazine (2 mg/kg) and atropine (0.05 mg/kg)) or by a suitable inhalant. A standard dose of 10$^7$ EID50/TCID50 (as appropriate) (10$^6$, if the higher dose is not possible) in 1 ml phosphate-buffered saline is slowly administered into the nares of the sedated animal, making sure that the virus is inhaled and not swallowed or expelled. A group of 4–6 ferrets should be infected. One group of ferrets (2–3 animals) should be killed on day 3 or 4 post-infection and the following tissues should be collected for estimation of virus replication: nasal turbinates and/or swabs, lung (tissue samples from each of four lobes and pooled), brain (tissues from anterior and posterior sections sampled and pooled), spleen and intestine. Additional lung tissue may be collected and processed for haematoxylin and eosin staining for microscopic evaluation of histopathology. The remaining animals are observed for 14 days for signs of weight loss, lethargy (based on a previously published index ($I$)), and respiratory and neurological symptoms. Neurological involvement may be confirmed by collection of brain tissue on day 14 post-infection at the termination of the experiment and processing as above for histopathology.

Expected outcome

Viral titres of the vaccine strain in respiratory tissues should be no greater than in either parental strain; a substantial decrease in lung virus replication is anticipated. Replication of the vaccine candidate should also be restricted to the respiratory tract and replication in the spleen or intestine is not expected. Although isolation of the vaccine strain from the brain is not desirable, if high viral titres are found in the nasal turbinates, there may be some detection of virus in the brain based on previous results with non-
virulent human H3N2 viruses (2). The significance of such a finding may be confirmed by performing a histopathological analysis of brain tissue on day 14 post-infection. Neurological lesions detected in hematoxylin and eosin-stained tissue sections confirm virus replication in the brain. Neurological symptoms and histopathology would indicate a lack of suitable attenuation of the vaccine candidate. Likewise clinical signs of disease such as weight loss and lethargy would indicate lack of attenuation in the vaccine strain, assuming that the wild-type avian virus also causes these symptoms.

Mice

Experimental procedure

The 50% lethal dose (LD50) of the vaccine strain and parental virus strains is determined in 6–8 week old female BALB/c mice. Mice are lightly anaesthetized with an inhalant and groups of mice (4–8 per group) are infected intranasally with 0.05 ml of serial 10-fold dilutions of virus (expected dose range 10^7 to 10^1 EID50). Mice are observed daily for disease signs and the numbers of deaths at each virus dilution are recorded. The LD50 values are calculated by the method of Reed and Muench (3). An additional three mice infected with a high dose of virus (e.g. 10^6) are killed on day 3 or 4 post-infection and organs, including the lungs and brain, are harvested for estimation of virus replication.

Expected outcome

If the wild-type avian strain replicates in the brain and is highly lethal for mice, the vaccine candidate should exhibit at least a 1000-fold reduction in LD50 values. Titres of the vaccine strain in lung and brain should be lower than those of either parental strain, consistent with an attenuation of replication in mouse tissues.

References

Annex 6

Recommendations for whole-cell pertussis vaccine

These Recommendations provide information and guidance to national regulatory authorities and vaccine manufacturers concerning the characterization, production and control of whole-cell pertussis vaccines to facilitate their international licensure and use. Each of the following sections constitutes a recommendation. The parts of each section printed in large type have been written in the form of requirements so that if a national regulatory authority so desires these parts may be adopted as definitive national requirements. The parts of each section printed in small type are comments and recommendations for guidance. It is recommended that modifications to these Recommendations be made only on condition that the modifications ensure that the vaccine is at least as safe and efficacious as that prepared in accordance with the Recommendations set out below. It is desirable that the World Health Organization should be kept informed of any such differences.

To facilitate the international distribution of vaccine made in accordance with these recommendations, a summary protocol is given in the Appendix.

Introduction
General considerations
Part A. Manufacturing recommendations
  A.1 Definitions
  A.2 General manufacturing recommendations
  A.3 Control of vaccine production
  A.4 Filling and containers
  A.5 Control of final product
  A.6 Records
  A.7 Samples
  A.8 Labelling
  A.9 Distribution and shipping
  A.10 Stability, storage and expiry date
Part B. Nonclinical evaluation of whole-cell pertussis vaccines
Part C. Clinical evaluation of whole-cell pertussis vaccines
Part D. National Control Recommendations
  D.1 General
  D.2 Official release and certification

Authors
Acknowledgements
References
Appendix
Summary protocol
Introduction

The WHO Requirements for whole-cell pertussis vaccine were first formulated in 1963 and the requirements for diphtheria and tetanus vaccines were prepared in 1964. Since diphtheria, tetanus and whole cell pertussis (DTwP) vaccines have been commonly used in a combined form, the requirements revised in 1978 contained separate sections on all three components as a followed by a final section that applied to a combination. The last revision of the requirements for diphtheria, tetanus, pertussis and combined vaccines was made in 1989 and published in 1990 (1).

Since that time a number of developments have taken place in the production, standardization and quality control of DTwP vaccines, as well as in the understanding of *Bordetella pertussis*, and it was considered that the existing requirements should be reviewed and where appropriate revised and updated. An amendment to the diphtheria and tetanus sections concerning single dilution and in vitro potency assays was adopted by the Expert Committee on Biological Standardization in 2004 (2). The present revision of the requirements for whole-cell pertussis vaccine should therefore be considered as part of the revision of the overall requirements for DTP. In 1998 the title WHO Requirements was changed to WHO Recommendations to better reflect the nature of these documents. These recommendations for whole-cell pertussis vaccine supersede those published in 1990 (1) and should be read in conjunction with the recommendations for diphtheria and tetanus vaccines when whole-cell pertussis vaccine is part of DTwP combined vaccine (1, 2). Once both the diphtheria and tetanus sections have also been fully revised, it is the intention to combine all three sections into one document.

Since 1989 a variety of combination vaccines based on DTP and involving a number of additional antigens have been developed and licensed. Many countries have already included tetravalent and pentavalent vaccines containing hepatitis B, *Haemophilus influenzae* type b conjugate (Hib) and inactivated polio vaccines (IPV), in addition to DTwP, in their immunization programmes. A need for further guidance on the evaluation of combination vaccines based on DTwP has been recognized and will be considered as a separate document.

In addition, a number of acellular pertussis vaccines have been licensed and used in combination with other vaccines for more than 20 years. Separate Guidelines for the production and control of the acellular pertussis component of monovalent or combined vaccines were developed in 1996 (3) (and their revision is also being undertaken separately).

General considerations

Pertussis is an important cause of infant death worldwide and continues to be a public health concern even in countries with high vaccination coverage.
Recent estimates from WHO suggest that in 2002 about 18,351,000 cases of pertussis occurred worldwide, the vast majority in developing countries, and that about 294,000 of those infected died. It is further estimated that in 2002, global vaccination against pertussis averted more than 37 million cases and 587,000 deaths (WHO/IVB database at http://www.who.int/immunization_monitoring/burden/estimates_burden/en/index.html).

It is clear that immunization programmes with high coverage have significantly reduced mortality and morbidity from the disease in many countries. However, despite its efficient prevention of clinical disease, the vaccine appears to have had limited impact on the circulation of *B. pertussis* even in countries with high vaccination coverage. The impact of vaccines on the circulating strains is not fully understood. In addition, during the 1990s, a significant epidemiological shift towards higher incidences of pertussis among schoolchildren previously vaccinated, adolescents and adults has been observed in many industrialized countries (4). This led to the consideration of a potential need for immunization of adolescents and adults to improve current control of whooping cough.

The optimal immunization schedule and the appropriate time for booster dose of DTwP vaccine should be assessed in individual national programmes taking into account the current epidemiological situation (4). Careful epidemiological surveillance of pertussis is encouraged worldwide to monitor disease burden and the impact of vaccination and particularly to compare different products and vaccination schedules.

Also, a shift in the antigenic properties of *B. pertussis* strains in circulation has been reported (5–7) and the continued monitoring of its potential impact on the overall immunity of a population is crucial in controlling the disease. Therefore, monitoring of genetic and antigenic characteristics of the pathogen in the context of the appropriateness of the strains of *B. pertussis* used in the production of both whole cell and acellular pertussis vaccines is encouraged.

Whole-cell pertussis vaccines have been used worldwide as part of combined DTP vaccine in national childhood immunization programmes for decades. Although concerns about possible adverse events following their administration have led to the adoption of acellular pertussis vaccines in some countries, whole-cell pertussis vaccines are still widely produced and used globally in both developed and developing countries. Whole-cell pertussis vaccines that comply with WHO requirements, administered according to an optimal schedule have a long and successful record in the control of whooping cough. Furthermore, the excellent efficacy of some currently available whole-cell pertussis vaccine has also been shown, not only in recent clinical trials, but also on the basis of the resurgence of disease where vaccination has been interrupted or when coverage has markedly decreased.
(8). Therefore, WHO continues to recommend whole-cell pertussis vaccines for use in national immunization programmes. Further details are available in a WHO position paper on pertussis vaccines (4).

In terms of severe adverse events, acellular pertussis and whole-cell pertussis vaccines appear to have acceptable safety, whereas mild to moderate adverse reactions are more commonly associated with the whole-cell pertussis vaccine. The latter is not recommended for use in adolescents and adults. So far, no clinically significant immunological interference has been documented between whole-cell pertussis vaccines and other antigens when they are offered in a combination formulation, or with other vaccines simultaneously administered at different injection sites. This is in contrast to the reduced antibody levels to Hib vaccine that have been observed when given in combination with some acellular pertussis vaccines (9).

Recent developments in the production, standardization and quality control of pertussis vaccines were reviewed by a Center for Biologics Evaluation and Research (CBER)/WHO working group on pertussis vaccines in November 2000 and at a WHO consultation in July 2003. However, the scientific basis for the present revision of the requirements for whole-cell pertussis vaccines was developed at a WHO consultation of national regulatory authorities, vaccine manufacturers and other experts, in March 2005. Key areas covered included vaccine composition, potency evaluation and toxicity testing.

Considerable progress has been made in understanding the nature of some of the agglutinogens of *B. pertussis* (10). These are surface proteins which, on infection, elicit the production of antibodies that cause the agglutination of the organism in vitro. Some have been identified as fimbriae. The presence of fimbriae 2 and 3, formerly identified as agglutinogens 2 and 3, in whole-cell pertussis vaccines is believed to contribute to their protective efficacy, and a test has been included in these revised Recommendations for the purpose of determining whether fimbriae 2 and 3 are present, before adjuvant is added.

The evidence that vaccines shown to protect mice against intracerebral challenge also protected immunized children against whooping cough when such children were exposed to the disease in the home by infection from a sibling was published in the 1950s. This correlation was the basis for the establishment of the current potency test (11). Although the potency test has a long record of use, it has often been criticized, especially on its reproducibility. However, a recent WHO proficiency study involving 13 laboratories in 12 countries confirmed that the intracerebral challenge assay was effective in distinguishing potent and sub-potent batches of vaccine and gave consistent results both between repeat tests and between different laboratories (12).
Nevertheless, the mouse protection test is technically demanding and efforts have been made to develop alternative in vitro potency assays, such as serological assays. However, the lack of understanding of the mechanisms of protection in humans afforded by whole-cell pertussis vaccines, in particular, of the value of neutralizing antibodies, the nature of the critical antigens and the role of cell-mediated immunity, it is difficult to design an acceptable alternative. The serological approach was extensively discussed at an European Directorate for the Quality of Medicines/European Centre for the Validation of Alternative Methods (EDQM/ECVAM) consultation in 2005 (13) where the issue of the relevance of simple antibody measurements to human clinical protection was considered. It was concluded that such tests cannot yet be considered as validated alternatives to the mouse protection potency test for whole-cell pertussis vaccines. However, correlation between production of agglutinins in mice and protection in children demonstrated as early as the Medical Research Council (MRC) trials in the 1950s should be further explored as a potential alternative or a complementary test to the currently recommended potency test. There was also a strong recommendation from the EDQM/ECVAM consultation to use validated humane end-points in the mouse protection test.

The use of the WHO Opacity Standard has also been much discussed. Comments from many manufacturers and discussion at the WHO Consultation in 2005 indicated that the estimation of the number of bacteria using the opacity of the bacterial suspension prior to inactivation is still a valuable parameter in the in-process control of whole-cell pertussis vaccines. Manufacturers are encouraged to continue to express opacity in International Units and to specify the range of values for their own vaccine product.

The role of different toxins, such as pertussis toxin, heat labile (dermonecrotic) toxin, tracheal cytotoxin, adenylate cyclase toxin and endotoxin in immunity to the natural infection or in immunization is not fully understood. A potential link between the presence of some of these toxins and reactogenicity in humans has been reported, but the mechanisms of their action and the contribution of individual toxins to overall toxicity remains unclear. Nevertheless, the determination of residual toxic activity remains an important aspect of the safety assessment. Residual levels of active pertussis toxin and endotoxin are likely to be a major contributor to the reactogenicity of whole-cell pertussis vaccines in humans and limits have been established for active pertussis toxin in acellular pertussis vaccines. The First International Standard for pertussis toxin has been established and various methods for the determination of residual levels of this toxin in vaccine preparations have been developed. At present, there is no scientific basis for setting specifications for pertussis toxin and endotoxin in whole-cell pertussis vaccine preparations, but monitoring their levels for consistency during production is encouraged.
In recent years, safety concerns have been raised over the use of thiomersal in vaccines, especially those given to infants. These concerns have been based primarily on data regarding the toxicity of a related substance, methyl mercury, and from data on chronic exposure to mercury via the food chain. Such safety concerns have led to initiatives in some countries to eliminate, reduce or replace thiomersal in vaccines, both in single dose and multidose presentations. It is important to note that the concerns about the toxicity of thiomersal are theoretical and there is no compelling scientific evidence of a safety problem with its use in vaccines, although a public perception of risk remains in some countries. WHO policy is clear on this issue, and the Organization continues to recommend the use of vaccines containing thiomersal for global immunization programmes because the benefits of using such products far outweigh any theoretical risk of toxicity (14). In the case of whole-cell pertussis vaccines, thiomersal has been used in the production process as an inactivating agent as well as a preservative. Potential changes in its content, following licensing, may affect quality, safety and efficacy of the vaccine. In the event of any change, WHO Guidelines on regulatory expectations related to the elimination, reduction or replacement of thiomersal in vaccines (15) should be followed.

**Changes made and issues addressed**

The main changes made, and issues addressed, in the present revision are as follows:

- Final vaccine bulk should be examined to ensure it contains predominantly phase I bacteria that display fimbriae 2 and 3. Strains of *B. pertussis* used in production should be well characterized emphasizing phase I organisms. Markers for phase I strains (e.g. haemolytic activity) are suggested in smaller print.
- The reference reagents currently available are listed and include reagents for the determination of fimbriae 2 and 3.
- Determination of bacterial concentration is considered to be an important in-process control test and the International Reference Preparation of Opacity is still considered to be a valuable tool.
- The recommendation to use the mouse weight gain test to assess specific toxicity of vaccine preparations has been upgraded to large print whereas the details of the methodology and refined methodology are displayed in smaller print.
- During monitoring of detoxification processes, as well as when validating methods used for detoxification and establishing consistency of production, manufacturers are encouraged to monitor levels of pertussis toxin and endotoxin. International Standards for pertussis toxin and endotoxin are available and results should be expressed in IU.
• The estimation of potency has been upgraded from small to large print clarifying that it should not be less than 4.0 IU per single human dose with a lower fiducial limit of the estimated potency being not less that 2.0 IU.
• Manufacturers and control laboratories are encouraged to use validated humane end-points in recording results of potency testing.
• A new section on the stability evaluation of vaccines has been included which emphasizes the importance of real-time studies under intended storage conditions and discusses the extent of stability studies needed for different purposes and at different stages of manufacturing.
• Specific issues for nonclinical and clinical evaluation of new pertussis vaccines as well as a need for the improvements in postmarketing surveillance are also discussed in separate sections of this document.

Part A. Manufacturing recommendations

A.1 Definitions

A.1.1 International name and proper name

The international name should be whole-cell pertussis vaccine. The proper name should be the equivalent of the international name in the language of the country of origin.

The use of the international name should be limited to vaccines that satisfy the recommendations formulated below.

A.1.2 Descriptive definition

Whole-cell pertussis vaccine is a suspension of the whole cells of one or more strains of killed *Bordetella pertussis* which have been appropriately treated to minimize toxicity and retain potency. The preparations for human use should satisfy all the recommendations formulated below.

A.1.3 International reference materials

The WHO catalogue of international biological standards should be consulted for the latest list of appropriate international standards and reference materials (http://www.who.int/biologicals/IBRP/Catalogue.htm).

The third International Standard for Pertussis Vaccine was established in 1998 with a potency of 46 IU of pertussis vaccine per ampoule.

The fifth International Reference Preparation of Opacity was established in 1975 with an opacity of 10 International Units. It consists of plastic rods simulating the optical properties of a bacterial suspension.

The WHO reference reagents of monoclonal antibodies for *B. pertussis* anti-fimbriae serotype 2 and 3 were established in 2004. They are intended for the determination of serotype of *B. pertussis* strains.
The First International Standard for Pertussis Toxin was established in 2003 with an activity of 10,000 IU per ampoule. It is intended for the determination of residual pertussis toxin in pertussis vaccine.

The above-mentioned International Standards/reference materials and other reagents from the WHO Pertussis reagent Bank are in the custody of the National Institute for Biological Standards and Control, Potters Bar, Hertfordshire, EN6 3QG, England (web site: http://www.nibsc.ac.uk).

These reference preparations are available for calibration and establishment of regional, national or in-house reference materials. Samples are distributed free of charge, on request, to national control laboratories.

A.1.4 **Terminology**

The following definitions are given for the purpose of these recommendations only.

*Seed lot.* A quantity of bacterial suspension that is derived from one strain, has been processed as a single lot and has a uniform composition. It is used for preparing the inoculum for the production medium.

*Single harvest.* A suspension of bacteria prepared from cultures of one strain of *B. pertussis* inoculated, harvested and processed together.

*Final bulk.* The homogeneous finished vaccine from which the final containers are filled either directly or through one or more intermediate containers.

*Final lot.* A collection of sealed final containers that are homogeneous with respect to the risk of contamination during filling. A final lot must therefore have been filled from a single container in one continuous working session.

A.2 **General manufacturing recommendations**

The general manufacturing recommendations contained in good manufacturing practices for pharmaceuticals (16) and biological products (17) should apply to establishments manufacturing whole-cell pertussis vaccine.

A.3 **Production control**

A.3.1 **Control of source materials**

A3.1.1 *Strains of Bordetella pertussis*

Strains of *B. pertussis* used in preparing vaccines should be identified by a full record of their history, including their origin, characteristics on isolation, and particulars of all tests made periodically to verify strain characteristics.
The strains should be well characterized and chosen in such a way that the final vaccine contains predominantly phase I cells that display fimbriae 2 and 3. They should have been shown to the satisfaction of the national regulatory authority, to yield safe and immunogenic vaccines when inactivated.

The reference preparations of antibodies for detection of fimbriae 2 and 3 should be used.

Since haemolytic activity has been suggested as a marker for phase I cells, colonies of *B. pertussis* can be examined for this characteristic on a suitable solid medium containing blood. Alternatively, some culture media (e.g. meat extract agar plates) support the growth of phase III/IV isolates of *B. pertussis*, but not that of phase I bacteria, and these media can be used to confirm phase I status of cultures. When culture methods for phase I assessment are used, the media composition, blood type and concentration, and incubation time need to be properly defined.

The strains should be maintained by a method that will preserve their ability to yield potent vaccine.

Freeze-drying or storage in liquid nitrogen is a satisfactory method of maintaining strains.

### A.3.1.2 Seed lot system

The production of pertussis vaccine should be based on a seed lot system. Cultures of the working seed should have the same characteristics as those of the strain from which the parent seed lot was derived.

### A.3.1.3 Culture media for production of bacteria

The media chosen for growing *B. pertussis* should be carefully selected and enable the organism to grow well and to retain phase I characteristics. Given that different media have an impact on the quality of the vaccine, every effort should be made to use media proved as a substrate for manufacturing a vaccine that consistently meets the potency requirements. Once the media have been demonstrated as appropriate they should be consistently used. Every change of media should be validated and the national regulatory authority notified.

The acceptability of the source(s) of any components of bovine, sheep or goat origin used in culture media should be approved by the national regulatory authority. *B. pertussis* 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 guidelines on medicinal and other products in relation to human and animal transmissible spongiform encephalopathies (18). When animal blood or blood products are used, they should be removed by washing the harvested bacteria.
In some countries, the use in the medium of blood from any source is not permitted. Manufacturers are encouraged to explore the use of media derived from non-animal sources.

Human blood or blood products should not be used in culture media for propagating bacteria, either for seed or for vaccine.

A.3.2 Control of single harvests

A.3.2.1 Monitoring consistency of production

Consistency of production should be demonstrated. Parameters to be measured include, but are not limited to, bacterial growth rate and some characteristics of phase I organisms in the culture, such as haemolytic activity and presence of fimbriae 2 and 3.

Criteria for acceptance or rejection of harvests should be defined.

A.3.2.2 Control of bacterial purity

Samples of single harvests taken before killing should be tested for purity by microscopic examination of stained smears or by inoculation into appropriate culture media. Single harvests should not be used for the final bulk if contamination has occurred at any stage in their production.

A.3.2.3 Control of opacity

The opacity of each single harvest should be measured not later than 2 weeks after harvesting and before the bacterial suspension has been subjected to any process capable of altering its opacity. It should be measured by comparison with the International Reference Preparation of Opacity or an equivalent reference preparation approved by the national regulatory authority. The opacity of bacterial suspensions should be expressed in International Units and specifications set for each vaccine.

A bacterial suspension having the same opacity as the International Reference Preparation of Opacity has a bacterial concentration providing 10 IU of opacity. The relationship between such units and actual numbers of bacterial cells may vary from vaccine to vaccine.

A spectrophotometric method validated against the opacity reference may also be used for this purpose.

A.3.2.4 Killing and detoxification

After samples of single harvests have been taken for purposes of purity control and opacity measurement, the bacteria shall be killed and detoxified by a method approved by the national regulatory authority. To ensure that the organisms have been killed, a sample should be tested in an appropriate culture medium.
B. pertussis can be killed by a number of methods whose effectiveness depends on the concentration of the chemicals used and the temperature, time and pH at which killing is carried out. The aim is twofold: to kill all bacterial cells and to achieve an appropriate level of detoxification without adversely affecting the potency or the physical characteristics of the vaccine. The methods used and kinetics of inactivation should be validated to the satisfaction of, and approved by, the national regulatory authority.

After killing and detoxification, the opacity of the suspension will be different from what it was originally. Each single harvest should, however, still be regarded as containing the same number of bacteria.

No biologically active heat-labile toxin (dermonecrotic toxin) should be detectable in a vaccine. The method of manufacture should be validated to ensure that no active dermonecrotic toxin is present in the final product. The method of detoxification should ensure vaccine safety. At present, it is not possible to recommend limits for levels of pertussis toxin, endotoxin, tracheal cytotoxin and adenylate cyclase in whole-cell pertussis vaccines. Manufacturers are encouraged to appropriately validate tests for these factors, and to ensure consistency of production.

A.3.3 Control of final bulk

A.3.3.1 Preparation

The final bulk may consist of a single harvest or a pool of single harvests from one or more strains. If a vaccine is prepared from two or more strains, the proportion of each strain in the pool, as calculated in opacity units, should remain consistent for each batch of the final bulk. The single harvest or pool should be diluted such that the number of bacteria in a single human dose of the final bulk is equivalent to the number of bacteria in the same volume of a suspension showing an opacity of no more than 20 IU. The opacity measured on the single harvests (before killing, see part A, section A.3.2.3) should be used to calculate the bacterial concentration in the final bulk.

A.3.3.2 Fimbriae

Each bulk should be examined, before adjuvant is added, for the presence of fimbriae 2 and 3 to ensure that appropriate expression has occurred during bacterial growth.

A.3.3.3 Preservative

If the vaccine is to be dispensed into multidose containers, a suitable antimicrobial preservative should be added. Consideration should be given to the effect of the preservative on stability of the vaccine formulation 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 national regulatory authority. The amount of preservative in the vaccine dose should be shown not to have any deleterious effect on the antigen nor impair the safety of the product in humans. The preservative, its use at different stages of the manufacturing process as well as its residual amount should be approved by the national regulatory authority.

If any modification of the preservative content in an already licensed vaccine is made, general principles for vaccine evaluation described in the WHO Guidelines on regulatory expectations related to the elimination, reduction or replacement of thiomersal in vaccines, should be followed (15).

Phenol should not be used as a preservative.

A3.3.4 Adjuvants
If an adjuvant has been added to the vaccine, its nature, purity and concentration should be determined by a method approved by the national regulatory authority.

Either aluminium or calcium compounds may be used as mineral carriers.

Where aluminium compounds are used as adjuvants the concentration of aluminium should not exceed 1.25 mg. When calcium adjuvants are used, the concentration of calcium should not exceed 1.3 mg per single human dose.

In some countries, an upper limit of 1.25 mg of aluminium is considered to be excessive for products containing a pertussis component and such vaccines therefore contain only 0.1–0.3 mg of aluminium per single human dose.

If other substances have been used as adjuvants or those with adjuvanted effect, specifications should be set and agreed by the national regulatory authority.

The formulation should be such that the homogeneous suspension appear after shaking and remains as such for a specified time (e.g. time needed for vaccine administration).

A3.3.5 Sterility
Each final bulk shall be tested for bacterial and fungal sterility in accordance with the requirements given in Part A, section 5, of the revised Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances) (19) or by a method approved by the national regulatory authority. If a preservative has been added to the
vaccine, appropriate measures should be taken to prevent it from interfering with the test.

A3.3.6 **Specific toxicity**

Each final bulk should be tested for toxicity using the mouse weight gain test. The final bulk is considered satisfactory if the following conditions are met:

(a) at the end of 72 hours the average weight of the group of vaccinated mice is not less than that preceding the injection,
(b) at the end of 7 days the average weight gain per mouse is not less than 60% of that per control mouse, and
(c) no deaths occur when 10 mice are used and no more than one death occurs when 20 mice are used.

A satisfactory method of carrying out the assay is as follows: at least 10 healthy mice each weighing 14–16 g are used for each vaccine group and for the saline control group. Mice should be of the same sex or segregated males and females should be distributed equally between all groups. Mice should have access to food and water for at least 2 h before injection, and continuously after injection for the duration of the test. The total weight of each group of mice should be measured immediately before injection. Each mouse is given an intraperitoneal injection of 0.5 ml of 0.85% NaCl aqueous solutions containing half of the recommended single human dose. The mice in the control group are inoculated with 0.5 ml of physiological saline, preferably containing the same amount of preservative as the inoculum injected into the test mice. The total weight of each group of mice is measured or calculated at 72 h and again at 7 days after injection.

If vaccine fails to meet the requirements in a first test, it can be retested once, and the results of the two valid tests should be combined.

If the average weight gain per mouse in the vaccine group is greater than 150% of that per control mouse, ascites production should be suspected and the test should be considered invalid.

Manufacturers are encouraged to develop refinements and alternatives to the mouse weight gain test.

In some countries a refinement of the mouse weight gain test is used. Mice are weighed individually immediately before injection, and 16–24 h, 72 h and 7 days after injection. On day 7 blood samples are taken from the tail vein and leukocytes are counted. Weight change at 16–24 h is considered to reflect the presence of lipo-oligosaccharide and an increase in the leukocyte count is considered to reflect the presence of pertussis toxin in the vaccine.
Other tests:
Cell harvests of *B. pertussis* to be used in the manufacture of pertussis vaccine contain a number of biologically active molecules which may contribute to the toxicity of the final product. Assays for some of these substances can be used to monitor and validate the methods used for detoxification and may also be useful in assessing final products. In the process of validating the manufacturing procedures, manufacturers are encouraged to monitor the following:

*Pertussis toxin.* A Chinese hamster ovary cell (CHO-cell) assay, based on the clustering of cells after treatment with pertussis toxin is used in some countries to measure pertussis toxin in vaccine. A pertussis toxin standard is included in the assay, and a vaccine reference is used as a positive control. All samples are serially diluted to obtain an end-point and the concentration of the pertussis toxin in the test sample is calculated in relation to the toxin reference. Tests for histamine sensitizing activity in mice may also be used.

*Endotoxin.* *B. pertussis* is a Gram negative organism, thus whole-cell pertussis vaccines contain lipo-oligosaccharide endotoxin. The endotoxin content of vaccines can be estimated by the limulus amoebocyte lysate assay or the rabbit pyrogen test. The limulus amoebocyte lysate assay is preferred. Although there is no agreement as to what constitutes an acceptable level of endotoxin in whole-cell pertussis vaccines, monitoring of endotoxin level on a lot-to-lot basis is encouraged as a monitor of consistency of production.

A3.3.7 Potency
The potency of each final bulk (or of each final lot) should be determined by comparison with that of a reference vaccine calibrated against the International Standard for Pertussis Vaccine or an equivalent standard vaccine approved by the national regulatory authority. The assay should be performed by the intracerebral mouse protection test. The assay method and the method of calculating the results should be approved by the national regulatory authority. The potency is estimated in terms of IU in the volume recommended for a single human dose. The vaccine passes the recommendations for potency if the result of a statistically valid test shows that the estimated potency of the vaccine is not less than 4.0 IU in the volume recommended for a single human dose and if the lower fiducial limit \((P = 0.95)\) of the estimated potency is not less than 2.0 IU. Additional tests may be done, but in this case the results of all valid tests must be combined in the weighted geometric mean estimate and its lower fiducial limit.

In some countries, an upper limit of potency is also specified.

A satisfactory method of carrying out the assay is as follows:
Mice. Healthy mice from a strain and colony capable of giving an adequate immune response are used. They should preferably be of the same sex but, if this is not possible, both sexes should be distributed equally throughout the test and the sexes segregated. Mice should be consistent for age and weight. An example of a criterion for consistency which has been used is that mice should weigh at least 10 g and not more than 18 g and in a single test the weight of the mice should not differ by more than 4 g.

The mice are randomly allocated to the different groups, and the shelf position of the cages, the order of immunization, and the order of challenge are also randomized. Groups of at least 16 mice should be used for each dilution of the standard vaccine and of the vaccines under test, and at least 10 mice should be used for each dilution of the culture in the estimation of the number of median lethal doses (LD$_{50}$) in the challenge dose.

Immunization of mice. At least three dilutions of the reference vaccine and of each lot of vaccine should be tested. Serial dilutions, not greater than fivefold, of the vaccine to be tested and of the standard vaccine should be made in a suitable diluent. The median effective dose (ED$_{50}$) for each preparation should be tested by the dilutions used. Each mouse in each immunization group should be injected intraperitoneally with 0.5 ml of the appropriate dilution.

The interval between immunization and challenge should be 14–17 days. At least 94% of the mice immunized by each dilution of both the reference vaccine and the test vaccines should survive until challenged, and each mouse challenged should appear healthy prior to challenge.

The challenge. The strain used for challenge (generally B. pertussis 18323) should be approved by the national regulatory authority. To ensure consistency of virulence from test to test, a large working challenge lot prepared from the master culture is dispensed into ampoules and freeze-dried or stored in liquid nitrogen.

The bacterial suspension used for challenge is prepared from a 20–24 h culture grown on Bordet–Gengou medium, or other suitable medium that has been seeded from a rapidly growing culture not more than 30 h old. Alternatively, aliquots of the challenge suspension may be frozen and kept in liquid nitrogen; after thawing and dilution, they can be used directly as the challenge culture. The suspension is diluted with a diluent in which the organisms will remain viable, e.g. an aqueous solution containing 10 g/l casein peptone and 6 g/l sodium chloride adjusted to a pH of 7.1 ± 0.1. The suspension, free from particles of agar or clumps of bacteria, is adjusted in such a way that each challenge dose of not more than 0.03 ml contains 100–1000 times the LD$_{50}$.
Mice immunized with the reference vaccine and the test vaccines are challenged at random under mild narcosis by intracerebral injection of the challenge dose. To obtain an estimate of the LD$_{50}$, dilutions of the challenge dose are then injected into control mice by the intracerebral route and an appropriate dilution of the challenge dose is cultured on Bordet–Gengou medium to determine the number of colony-forming units contained therein.

Recording of results. The mice are observed for 14 days. Mice that die within 72 hours should be excluded from the test. To determine the ED$_{50}$ of the vaccines, records should be kept of the number of mice that die after 72 hours. Animal welfare regulations should be followed.

The use of validated humane end-points is encouraged.

**Calculation of results.** The ED$_{50}$ values for each preparation are determined by a statistical method that includes the transformation of the mouse survival data into a form capable of consistently producing a linear regression. Probits, logits and angle transformation have been shown to be suitable. Similar methods should be used to determine the LD$_{50}$ of the challenge suspension.

**Validity of the test.** The test is valid if the ED$_{50}$ of each vaccine is intermediate between the largest and the smallest immunizing doses, and the regressions do not show significant deviation from linearity and parallelism ($P < 0.05$). The challenge dose should contain 100–1000 LD$_{50}$ and the LD$_{50}$ should contain no more than 300 colony-forming units.

**Estimate of potency.** The ED$_{50}$ of the vaccine under test and the standard vaccine are calculated by a method that provides an estimate of the limits of the 95% confidence intervals. The potency is estimated in terms of IU in the volume recommended for a single human dose.

A.3.3.8 pH

The pH of each final bulk should be measured and specifications set.

In some countries this test is applied to the final filled vaccine (A 5.7).

A.4 **Filling and containers**

The requirements concerning filling and containers given in *Good manufacturing practices for biological products* (17) should apply to vaccine filled in the final form.

Single-dose or multiple-dose containers may be used. Vaccine in multidose containers should contain a suitable antimicrobial preservative.
A.5  **Control of pertussis component in final lot**

A.5.1  **Identity**

An identity test should be performed on at least one container from each final lot.

The identity test may be based on an immunological reaction (for example, agglutination of the organisms) with a specific antipertussis serum. Alternatively, vaccines may also be inoculated into animals to show that pertussis-specific antibodies (e.g. agglutinins) are present in their serum.

A.5.2  **Sterility**

Final containers should be tested for sterility by a method approved by the national regulatory authority.

Many countries have regulations governing the sterility testing of the final product. Where these do not exist, the requirements published by WHO should be met \(^{(19)}\). If a preservative has been added to the vaccine, appropriate measures should be taken to prevent it from interfering with the sterility test.

A.5.3  **Potency**

A potency test should be carried out as provided in Part A, section A.3.3.7, on each final lot, if such a test has not been done on the final bulk.

A.5.4  **General safety (innocuity) test**

Each final lot should be tested for unexpected toxicity (sometimes called abnormal toxicity) using a general safety (innocuity) test approved by the national regulatory authority.

This test may be omitted for routine lot release once consistency of production has been well established to the satisfaction of the national regulatory authority and when good manufacturing practices are in place. Each lot, if tested, should pass a test for general safety.

A.5.5  **Adjuvant content**

If an adjuvant has been added to the vaccine, its content should be determined by a method approved by the national regulatory authority.

When aluminium compounds are used as adjuvants, the concentration of aluminium should not exceed 1.25 mg per single human dose. If a calcium adjuvant is used, the concentration of calcium should not exceed 1.3 mg per single human dose.

If other substances were used as adjuvants, appropriate specifications should be set for the substance with adjuvant effect.
A.5.6 *Preservative content*

If a preservative has been added to the vaccine, the content of preservative should be determined by a method approved by the national regulatory authority.

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, its use at different stages of the manufacturing process as well as its residual amount should be approved by the national regulatory authority.

If any modification of thiomersal content in an already licensed vaccine is made, general principles for vaccine evaluation described in the WHO Guidelines on regulatory expectations related to the elimination, reduction or replacement of thiomersal in vaccines, should be followed (15).

A.5.7 *pH*

The pH of each final lot should be measured and specifications set.

In some countries this test is applied to the final bulk only (A 3.3.8).

A.5.8 *Inspection of final containers*

Each container in each final lot should be inspected visually, and those showing abnormalities — such as improper sealing, lack of integrity, clumping or the presence of particles — should be discarded.

A.6 *Records*

The recommendations given in Good Manufacturing Practices for biological products (17) should apply.

A model of a suitable summary protocol to be used for pertussis vaccines is given in the Appendix.

A.7 *Retained samples*

The recommendations given in Good Manufacturing Practices for biological products (17) (Annex 1) should apply.

A.8 *Labelling*

The recommendations given in Good Manufacturing Practices for biological products (17) should apply, with the addition of the following.

— the words whole-cell pertussis vaccine;
— the word “adsorbed”, if applicable;
— the name and address of the manufacturer;
— the recommended storage temperature and the expiry date if kept at that
temperature; and
— the recommended single human dose and route of administration.

In addition, the label printed on or affixed to the container, or the label
on the carton, or the leaflet accompanying the container shall contain the
following:

— a statement that the vaccine satisfies the requirements of this document;
— the nature and amount of any preservative present in the vaccine
(if there is no preservative in single-dose containers, this should be
stated);
— the nature and amount of the adsorbing agent, if applicable;
— the nature and amount of any substances added to the vaccine;
— the recommended conditions for storage and transport;
— a warning that the vaccine should not be frozen;
— a warning that the vaccine should be shaken before use; and
— instructions for the use of the vaccine and information on contraindications
and the reactions that may follow vaccination.

A.9 Distribution and transport

The recommendations given in Good Manufacturing Practices for biological
products (17) should apply.

A.10 Stability, storage and expiry date

A.10.1 Stability

Stability evaluation is an important part of the quality assessment. The
purpose of stability studies is to ensure that the vaccine at the end of its shelf
life, storage period or period of use, still has the required characteristics
supporting quality, safety and efficacy.

For licensing

Studies that support stability of a vaccine for the purpose of licensing have
to be performed as real-time studies under the intended storage conditions.
Stability-indicating parameters should be carefully selected. They should
always include, but should not be limited to, the potency test. Tests should
be conducted to determine the loss of potency at appropriate time intervals
during storage. Final containers from at least three batches of vaccine derived
from different bulks should be tested on the expiry date to demonstrate
stability during storage.

Accelerated stability data for product stored for limited periods at
temperatures that may affect stability could support preliminary data
from ongoing real time stability studies but should not replace them. Any modification of the shelf life approved as part of licensing requires additional stability data to support the proposed modification and should be approved by the national regulatory authority. Following licensure, stability should be monitored throughout the proposed shelf-life.

**At different stages of manufacturing process**

Stability testing should be performed at different stages of production, namely single harvests, final bulk and final lot. Stability indicating parameters should be selected according to the stage of production. Manufacturers are encouraged to assign a shelf-life to all materials during vaccine production, in particular to intermediates such as single harvests, purified bulk and final bulk.

**For clinical trial approval**

For vaccines under development, stability data, such as those described above, are expected for the purpose of clinical trial approval. However, the stability data for such vaccines are generally available for a limited period.

Appropriate documentation to support the stability profile of a vaccine should be submitted to the competent national regulatory authority at all stages mentioned above.

**A.10.2 Storage conditions**

Recommended storage conditions and defined maximum duration of storage should be based on stability studies as described in section 10.1 above and approved by the national regulatory authority. For pertussis vaccines, a temperature of 2–8 °C has been found satisfactory. This should ensure that the minimum potency specified on the label of the container or package will still be maintained after release until the end of the shelf-life, if the conditions under which the vaccine is stored are in accordance with what is stated on the label.

The manufacturer should recommend conditions of storage and transport that will ensure that the vaccine satisfies the potency requirements until the expiry date stated on the label.

The vaccine must not be frozen.

**A.10.3 Expiry date**

The expiry date should be defined on the basis of the shelf-life supported by the stability studies as described above (section 10.1) and approved by the national regulatory authority.
Part B. Nonclinical evaluation of whole-cell pertussis vaccines

Nonclinical evaluation of new pertussis vaccines

For a new whole-cell pertussis vaccine, a new formulation, or for a vaccine produced by a manufacturer with no previous experience of such vaccines and which has not been previously tested in humans, proof of concept in a relevant animal model in terms of both potency and safety should be demonstrated. In addition, a safety assessment should be undertaken before initiation of the clinical evaluation of a new vaccine. General principles for the design, conduct, analysis and evaluation of nonclinical data are available in the WHO guidelines for nonclinical evaluation of vaccines (20). In particular, studies on safety pharmacology intended to investigate the effects of a vaccine on vital functions should be undertaken.

Part C. Clinical evaluation of whole-cell pertussis vaccines

New whole-cell pertussis vaccines, vaccines with a new formulation, or those intended to use a new route of administration and/or produced by a manufacturer with no previous experience with such vaccines should undergo clinical evaluation. This section is intended to indicate some of the specific issues which need to be considered in the clinical testing of such vaccines, as monocomponent vaccines or as a part of a combination. Issues to be considered in designing clinical studies for licensing as well as those for monitoring clinical effectiveness and safety in postmarketing surveillance studies are discussed.

In general, clinical trials should adhere to the principles described in good clinical practice (21) as well as to those formulated for the design, conduct and analysis of vaccine clinical trials described in the WHO guidelines for clinical evaluation of vaccines (22). Data generated in clinical trials should be submitted to the national regulatory authority as described in the Summary protocol for vaccine evaluation (22). All clinical trials should be approved by the relevant national regulatory authority.

However, there are issues which apply specifically to pertussis clinical trials and these should be considered in addition to the general principles mentioned above. First, prospective randomized controlled studies of protective efficacy (i.e. testing against a placebo) cannot be performed for ethical reasons. Second, trials designed to measure efficacy relative to that of a licensed whole-cell pertussis vaccine with proven efficacy, would need to be very large in order to provide adequate precision in the efficacy estimates.
An additional complexity is that many different antigens are expressed by *B. pertussis* and there are many different assays that might be used for the assessment of the immunogenicity. However, without any established immunological correlate(s) of protection the data cannot be used to predict efficacy.

C.1 **Clinical evaluation of new whole-cell pertussis vaccines for licensing**

C.1.1 **Compliance with the recommendations for production and control**

Candidate vaccine should comply with the recommendations for production and control described in part A of this document.

C.1.2 **Immunogenicity and safety assessment in humans**

C.1.2.1 A comparability study using a “new” wP preparation which meets these requirements for potency and safety, and a wP-containing vaccine that has been licensed for some years and used extensively in countries with reliable postmarketing safety surveillance schemes may be an appropriate approach.

C.1.2.2 The immune response in clinical trials should be assessed by using a small range of validated assays. Selection of the assays for evaluation of the immune response to the vaccine should be justified by the vaccine developer; when feasible, assays that measure functional immune responses should be employed. The assays used are unlikely to be commercially available, and thus validation issues must be addressed.

C.1.2.3 For each assay used, the immunogenicity data obtained should be compared both in terms of percentage of vaccinees who demonstrate a response (e.g. the percentage who reach a specified threshold or achieve a significant increase in antibody concentration) and in geometric mean concentrations (GMCs).

C.1.2.4 Every effort should be made to determine antibody response to individual, specific antigens rather than relying solely on the measurement of antibodies against whole cells or whole-cell extracts. Because of the historical link to clinical efficacy, the measurement of whole-cell agglutinins is recommended. Additionally, at least one assay used should determine antibodies against pertussis toxin.

C.1.2.5 The size of such a study and the end-points for evaluation require justification. The immunogenicity end-points need to be set according to the sensitivity and specificity of the assays and in the light of experience regarding natural variation between individuals.

C.1.2.6 In the case of combination with other antigens, potential interactions between the whole cell pertussis component and the others
C.1.2.7 Safety assessment should be part of the comparability study mentioned above with defined objectives of the study. The study should have sufficient power to provide reliable rates of frequent or very common adverse events (22).

C.1.2.8 The rates of specific adverse events should be formally compared: the non-inferiority margin should be based on anticipated rates from the trials conducted in the past.

C.2 Monitoring vaccine effectiveness and safety in the population

Every effort should be made to improve current scientific understanding of the protection in humans by providing data from active postmarketing surveillance.

Vaccine effectiveness in the population should be reported wherever possible.

Given that limited safety data are obtained in pre-licensure studies, all relevant safety indicating parameters should be monitored as part of postmarketing surveillance.

Data generated in postmarketing surveillance should be submitted to the national regulatory authority.

Part D. Recommendations for National Regulatory Authorities

D.1 General

The general recommendations for National Regulatory Authorities contained in the Guidelines for National Authorities on Quality Assurance for Biological Products (23) should apply.

The detailed production and control procedures and any change in them that may affect the quality, safety or efficacy of whole-cell pertussis vaccine should be discussed with and approved by the National Regulatory Authority. The National Regulatory Authority should establish a national working reference preparation calibrated against the International Standard for Pertussis Vaccine.

Consistency of the production has been recognized as an essential component in the quality assurance of whole-cell pertussis vaccines. In particular,
National Regulatory Authority should carefully monitor results of tests performed on a series of consecutive batches of the final bulk.

**D.2 Official release and certification by the national regulatory authority**

A vaccine lot should be released only if it fulfills national requirements and/or satisfies Part A of these Recommendations.

A statement signed by the appropriate official of the national regulatory authority should be provided at the request of the manufacturing establishment and should certify that the lot of vaccine in question satisfies all national requirements as well as Part A of the present Requirements. The 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. The official national release document should be provided to importers of pertussis vaccines.

The purpose of the certificate is to facilitate exchange of pertussis vaccines between countries. A model of a suitable certificate is given in the appendix.

**Authors**

The scientific basis for the revision of the Requirements published in 1990 was discussed at the meeting of the working group held at the World Health Organization, Geneva, in July 2003 attended by the following people: Dr J. Arciniega, Center for Biologics Evaluation and Research, Rockville, MD, USA; Dr M. Corbel, National Institute for Biological Standards and Control, Potters Bar, England; Dr R. Gaines-Das, National Institute for Biological Standards and Control, Potters Bar, England; Dr E. Griffiths, Centre for Biologics Research, Biologics and Genetic Therapies Directorate Health Canada, Ottawa, Canada; Dr J.G. Kreeftenberg, Netherlands Vaccine Institute (NVI), Bilthoven, The Netherlands; Dr S.S. Jadhav, Quality Assurance and Regulatory Affairs, Serum Institute of India Ltd, Pune, India; and Dr D. Xing, National Institute for Biological Standards and Control, Potters Bar, England.

The first draft of these Recommendations was prepared by Dr Ivana Knezevic, Quality Assurance and Safety of Biologicals, World Health Organization, Geneva, Switzerland and Dr Dorothy Xing, National Institute for Biological Standards and Control, Potters Bar, England, following discussion at the National Institute for Biological Standards and Control, in April 2004, attended by following participants: Dr Elwyn Griffiths, Health Canada, Canada; Dr Michael Corbel; Dr Dorothy Xing and Dr Rose Gaines-Das, National Institute for Biological Standards and Control, Potters Bar, England.

Taking into account comments on the first draft, a second draft was prepared by Dr Ivana Knezevic, Quality Assurance and Safety of Biologicals, World Health Organization, Geneva, Switzerland and Dr Elwyn Griffiths, Health Canada, Canada, in January 2005.
The third draft was prepared by Dr Rose Das; Dr Dorothy Xing and Dr Michael Corbel, National Institute for Biological Standards and Control, Potters Bar, England; Dr Yoshinobu Horiuchi, National Institute for Infectious Diseases, Japan; Dr Elwyn Griffiths, Health Canada, Canada, and Dr Ivana Knezevic, Quality Assurance and Safety of Biologicals, World Health Organization, Geneva, Switzerland, after an informal WHO Consultation held in March 2005, with the following participants: Dr J. Arciniega, Center for Biologics Evaluation and Research, Rockville, MD, USA; Dr C.M. Ausiello, Instituto Superiore di Sanita, Rome, Italy; Dr T.A. Bektimirov, L.A. Tarasevich State Research Institute for Standardization and Control of Medical Biological Preparations, Moscow, Russian Federation; Dr P. Chagnaud, French Health Products Safety Agency, Lyon, France; Dr M. Corbel, National Institute for Biological Standards and Control, Potters Bar, England; Dr A. Dias, Oswaldo Cruz Foundation/FIOCRUZ, Rio de Janeiro, Brazil; Dr R. Dobbeltaer, Scientific Institute of Public Health-Louis Pasteur, Brussels, Belgium; Dr R. Gaines-Das, National Institute for Biological Standards and Control, Potters Bar, UK; Dr G Gallegos Flores, Comision de Control Analitico y Amplificacion de Cobertura, Gerencia de Analisis y Desarrollo de Pruebas Biologicas, Mexico; Dr M. Girard, Centre for Biologics Research, Biologics and Genetic Therapies Directorate Health Canada, Ottawa, Canada; Dr E. Griffiths, Centre for Biologics Research, Biologics and Genetic Therapies Directorate Health Canada, Ottawa, Canada; Dr N. Guiso, National Centre of Reference of Pertussis, Pasteur Institute, Paris, France; Dr S.R. Gupta, Directorate General of Health Services, Ministry of Health and Family Welfare, Government of India, New Delhi, India; Dr Y. Horiuchi, Dept. of Bacterial Pathogenesis and Infection Control (NIID), Tokyo, Japan, Mrs T. Jivapaisarnpong, Ministry of Public Health, Thailand; Dr J.G. Kreeftenberg, Netherlands Vaccine Institute (NVI), Bilthoven, The Netherlands; Dr B. Meade, Office of Vaccines Review and Research, Center for Biologics Evaluation and Research, Rockville, MD, USA; Dr A. M'henni, Institut Pasteur de Tunis, Ministry of Public Health, Tunis, Tunisia; Dr P. Olin, Swedish Institute for Infectious Disease Control, Solna, Sweden; Dr M. Powell, Medicines and Healthcare products Regulating Agency, London, England; Dr A. Tahlan, Joint Director and Government Central Research Institute, Kasauli, India; Dr C. von Hunolstein, Instituto Superiore di Sanita, Rome, Italy; Dr D. Xing, National Institute for Biological Standards and Control, Potters Bar, England; Dr S. Zhang, National Institute for the Control of Pharmaceutical and Biological Products, State Food and Drug Administration, Beijing, People's Republic of China; Ms E. Molari, UNICEF Supply Division, Copenhagen, Denmark; Dr Ma Verónica Ortega Adame, Comision de Control Analitico y Amplificacion de Cobertura, Gerencia de Analisis y Desarrollo de Pruebas Biologicas, Mexico; Dr S.S. Jadhav, Quality Assurance and Regulatory Affairs, Serum Institute of India Ltd, Pune, India; Dr Y. Lingjiang, International Business and Co-operation, Chengdu Institute of Biological Products, Chengdu, People's Republic of China; Dr E. Ma Fajardo, Vaccine Adviser and International Affairs, Finlay Institute, Cuba; Dr M. Qin, Bacterial Vaccine Department, Chengdu Institute of Biological Products, Chengdu, People's Republic of China; Dr N. Harjee, CSL Consultant, Ontario, Canada; Dr B. t'Serstevens, GSK, Rixensart, Belgium; Dr E. Vidor, Sanofi Pasteur, Lyon, France; Dr M-E. Behr Gross, Strasbourg, France; Dr D. Wood, Coordinator, Quality Assurance and Safety of Biologicals, World Health Organization, Geneva, Switzerland; Dr Carmen Rodriguez Hernandez, Access to Technology, World Health Organization, Geneva, Switzerland; Dr Ivana Knezevic, Quality Assurance and Safety of Biologicals, World Health
Organization, Geneva, Switzerland; Dr Philippe Duclos, Vaccine Assessment and Monitoring, World Health Organization, Geneva, Switzerland.

The first draft of the section on clinical evaluation of whole-cell pertussis vaccines was prepared by Dr Mair Powel, Medicines and Healthcare Products Regulating Agency, London, England, and Dr Ivana Knezevic, Quality Assurance and Safety of Biologicals, World Health Organization, Geneva, Switzerland, following discussion on 17 March 2005 attended by the following participants: Dr N. Guiso, National Centre of Reference of Pertussis, Pasteur Institute, Paris, France; Dr B. Meade, Office of Vaccines Review and Research, Center for Biologics Evaluation and Research, Rockville, MD, USA; Dr P. Olin, Swedish Institute for Infectious Disease Control, Solna, Sweden; Dr C.M. Ausiello, Instituto Superiore di Sanita, Rome, Italy; Dr M. Powell, Medicines and Healthcare Products Regulating Agency, London, England; Dr E. Vidor, Sanofi Pasteur, Lyon, France; Dr Philippe Duclos, Vaccine Assessment and Monitoring, World Health Organization, Geneva, Switzerland and I. Knezevic, Quality Assurance and Safety of Biologicals, World Health Organization, Geneva, Switzerland. Taking into account comments and suggestions received from the participants at this consultation, the clinical section was finalized.

Acknowledgements

Thanks are due to the following experts for their comments and advice on these Guidelines: Dr S.S. Jadhav, Quality Assurance and Regulatory Affairs, Serum Institute of India Ltd, Pune, India; Dr E. Ma Fajardo, Vaccine Adviser and International Affairs, Finlay Institute, Cuba; Dr A. Tahlan, Joint Director and Government Central Research Institute, Kasauli, India; Dr J. Arciniega, Center for Biologics Evaluation and Research, Rockville, MD, USA; Dr B. Meade, Office of Vaccines Review and Research, Center for Biologics Evaluation and Research, Rockville, MD, USA; Dr M. Powell, Medicines and Healthcare Products Regulating Agency, London, England and Dr M-E. Behr Gross, Strasbourg, France.

References


Appendix

Summary protocol for whole-cell pertussis vaccine production and testing

Summary information on final lot

Name and address of manufacturer ______________________________
__________________________________________________________

Lot no. ____________________________________________________
Date of filling ______________________________________________
Date of manufacturing ________________________________________

Nature of final product (absorbed) ______________________________
Volume of each recommended single human dose __________________________
No. of doses per final container __________________________________
No. of final containers _________________________________________

Container/closure system for the final lot _________________________
Expiry date _________________________________________________

Detailed information on manufacture and control

Strain

Identity of B. pertussis strains used in vaccine ______________________
Serological types of strains ______________________________________
Reference no. of seed lot _________________________________________
Date(s) of reconstitution of ampoule(s) for manufacture ______________

Single harvests used for preparing final bulk

List the single harvests and indicate the medium, dates of inoculation, temperature of incubation, dates of harvests, volumes, results of tests for bacterial purity, methods and dates of inactivation, opacity and fimbriae present.

Final bulk

Identification _________________________________________________
Volume ______________________________________________________
No. of opacity units (calculated from opacities of single harvests) ________________________________
**Test for fimbrae 2 and 3**

Date and results (before addition of adjuvant) ________________________

**Sterility test**

Sample tested and volume ________________________________

Media, volume and temperatures of incubation

Date(s) of inoculation ________________________________

Date(s) of end of observation ________________________________

Result of each test ________________________________

**Specific toxicity test (mouse weight-gain test)**

Strain of mice ____________________________________________

No. of mice ____________________________________________

Volume and route of injection __________________________________

Date of end of observation ________________________________

Result of test: on a separate sheet of paper, give all relevant details on mice in the control and test groups (survival, mean weight on day of injection and three and seven days after injection) and indicate percentage weight of test group as compared with control group.

**Other specific toxicity tests**

Mention here date and results of any other specific toxicity test(s) which may have been performed (e.g. tests for heat-labile toxin, lymphocytosis promoting factor and endotoxin) __________________________________

**Potency test**

Strain, weight and sex of mice __________________________________

Date of immunization __________________________________

$LD_{50}$ in challenge dose __________________________________

No. of colony-forming units in challenge dose ________________________________

Date of challenge __________________________________

Date of end of observation __________________________________

Results __________________________________

Calculation method ________________________________

<table>
<thead>
<tr>
<th>Reference vaccine (.IU/ml)</th>
<th>Dilution</th>
<th>No. of survivors/ No. of animals inoculated</th>
<th>Median effective dose ($ED_{50}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>ml</td>
</tr>
</tbody>
</table>

330
<table>
<thead>
<tr>
<th>Dilution</th>
<th>No. of survivors/No. of animals inoculated</th>
<th>Median effective dose (ED$_{50}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test vaccine</td>
<td>_____________________</td>
<td>_________ ml</td>
</tr>
</tbody>
</table>

Potency of test vaccine is ... IU per single human dose. Limits of 95% confidence interval (in %) are ...

**pH**

Date of measurement _____________________  
Result _____________________  
Specification _____________________

**Final product**

Identity test _____________________  
Date of test _____________________  
Type of test and result _____________________

**Sterility test**

No. of times the test had to be performed _____________________  
No. of containers tested in each test and volume _____________________  
Media, volume and temperatures of incubation _____________________  
Date(s) of inoculation _____________________  
Date(s) of end of observation _____________________  
Result of each test _____________________

**Potency test**

If the test was not performed on the final bulk, indicate this and report the data obtained on the final product in the space provided for potency tests in the “final bulk” section.

<table>
<thead>
<tr>
<th>Innocuity test</th>
<th>Mice</th>
<th>Guinea-pigs</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of animals</td>
<td>_____________________</td>
<td>_____________________</td>
</tr>
<tr>
<td>Route of injection</td>
<td>_____________________</td>
<td>_____________________</td>
</tr>
<tr>
<td>Volume of injection</td>
<td>_____________________</td>
<td>_____________________</td>
</tr>
<tr>
<td>Date of start of test</td>
<td>_____________________</td>
<td>_____________________</td>
</tr>
<tr>
<td>Date of end of test</td>
<td>_____________________</td>
<td>_____________________</td>
</tr>
<tr>
<td>Results</td>
<td>_____________________</td>
<td>_____________________</td>
</tr>
</tbody>
</table>

**Test for adjuvant**

Date of test _____________________  
Nature and concentration of adjuvant per single human dose _____________________  
Method of testing _____________________
Test for preservative
Date of test _______________________________________________
Nature and concentration of preservative _______________________
Method of testing ____________________________________________
Specification _______________________________________________
Result ______________________________________________________

pH
Date of measurement _________________________________________
Method of testing ____________________________________________
Specification _______________________________________________
Result ______________________________________________________

Inspection of final containers
Date of inspection __________________________________________
Organoleptic characteristics __________________________________
Number of containers inspected ________________________________
% of rejected containers ______________________________________

Stability test\(^1\)
Indicate separately all relevant details and (as a percentage) the calculated losses of potency per year at different temperatures, as determined by accelerated degradation tests, and actual titres\(^2\) (with limits of 95% confidence intervals) after storage for the maximum period claimed for the product at the recommended temperature.

Certification by the manufacturer
Name of head and production (typed) __________________________

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 pertussis vaccine, whose number appears on the label of the final containers, meets all national requirements\(^3\) and satisfies Part A of the pertussis vaccine section of Requirements for Biological Substances Nos. 8 and 10, revised 1989 and (if applicable) addenda 19...

---

\(^1\) Not required in summary protocols of every batch.

\(^2\) Needed only for three batches to validate the production method.

\(^3\) If any national requirement(s) is (are) not met, specify which one(s) and indicate why release of the lot has nevertheless been authorized.
Signature __________________________________________________
Name (typed) _______________________________________________
Date ______________________________________________________

Certification by the national regulatory authority

If the vaccine is to be exported, attach a certificate from the national regulatory authority as shown in Appendix 2, a label from a final container, and an instruction leaflet for users.
Annex 7

Biological substances: international standards and reference reagents

A list of International Standards and Reference Reagents for biological substances was issued in WHO Technical Report Series, No. 897, 2000 (Annex 4) and an updated version is available on the Internet at http://www.who.int/biologicals. Copies of the list may be obtained from appointed sales agents for WHO publications or from: Marketing and Dissemination, World Health Organization, 1211 Geneva 27, Switzerland.

At its meeting in October 2005, the WHO Expert Committee on Biological Standardization made the following changes to the previously published list.

These substances are held and distributed by the International Laboratory for Biological Standards, National Institute for Biological Standards and Control, Potters Bar, Herts., EN6 3QG, England.

**Additions**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Activity</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antigens and related substances</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haemophilus influenza type b capsular polysaccharide</td>
<td>4.933 ± 0.267 mg/ampoule of polyribosyl ribitol phosphate (PRP)</td>
<td>First International Standard (2005)</td>
</tr>
<tr>
<td><strong>Antisera</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-dengue virus types 1, 2, 3 and 4 serum</td>
<td>100 units per serotype per ampoule</td>
<td>First WHO reference reagent (2005)</td>
</tr>
<tr>
<td>Anti-human platelet antigen-1a</td>
<td>100 IU per ampoule</td>
<td>First International Standard (2005)</td>
</tr>
<tr>
<td>Anti-A blood grouping minimum potency Reagent</td>
<td>No assigned activity; however a 1 in 8 dilution should define the recommended minimum potency specification for anti-A blood grouping reagents</td>
<td>First International Standard (2005)</td>
</tr>
<tr>
<td>Preparation</td>
<td>Activity</td>
<td>Status</td>
</tr>
<tr>
<td>------------------------------</td>
<td>---------------------------------------------------------------------------</td>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>Anti-B blood grouping</td>
<td>No assigned activity; however a 1 in 4 dilution should define the</td>
<td>First International Standard (2005)</td>
</tr>
<tr>
<td>minimum potency reagent</td>
<td>recommended minimum potency specification for anti-B blood grouping</td>
<td></td>
</tr>
<tr>
<td></td>
<td>reagents</td>
<td></td>
</tr>
<tr>
<td>Blood products and related substances</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prothrombin mutation</td>
<td>No assigned activity</td>
<td>First International Genetic Reference Panel (2005)</td>
</tr>
<tr>
<td>G20210A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coagulation factor V, plasma, human</td>
<td>0.74 IU of Factor V:C per amapoule</td>
<td>First International Standard (2005)</td>
</tr>
<tr>
<td>Coagulation factor XI, plasma, human</td>
<td>0.86 IU per amapoule</td>
<td>First International Standard (2005)</td>
</tr>
<tr>
<td>Thromboplastin, rabbit, plain</td>
<td>International Sensitivity Index (ISI) value of 1.15</td>
<td>Third International Standard (2005)</td>
</tr>
<tr>
<td>Cytokines, growth factors and endocrinological substances</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vascular endothelial growth factor, human</td>
<td>13 000 units per amapoule</td>
<td>First WHO Reference Reagent (2005)</td>
</tr>
<tr>
<td>Keratinocyte growth factor, human</td>
<td>4000 units per amapoule</td>
<td>First WHO Reference Reagent (2005)</td>
</tr>
<tr>
<td>Keratinocyte growth factor, (24-163), human</td>
<td>9000 units per amapoule</td>
<td>First WHO Reference Reagent (2005)</td>
</tr>
<tr>
<td>Diagnostic reagents</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Annex 8

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, 1211 Geneva 27, Switzerland.

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

Marketing and Dissemination
World Health Organization
1211 Geneva 27
Switzerland
Telephone: + 41 22 79 12 476
Fax: +41 22 79 14 857
e-mail: publications@who.int

Individual recommendations and guidelines may be obtained free of charge as offprints by writing to:

Quality, Safety and Standards
Department of Immunization, Vaccines and Biologicals
World Health Organization
1211 Geneva 27
Switzerland

---

1 Abbreviated in the following pages as TRS.
Recommendations, Guidelines and other documents

<table>
<thead>
<tr>
<th>Recommendations, Guidelines and other documents</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acellular pertussis component of monovalent or combined vaccines</td>
<td>Adopted 1996, TRS 878 (1998)</td>
</tr>
<tr>
<td>Animal cells, use of, as in vitro substrates for the production of biologicals</td>
<td>Revised 1996, TRS 878 (1998); Addendum 2003, TRS 927 (2005)</td>
</tr>
<tr>
<td>Biological standardization and control: a scientific review commissioned by the UK National Biological Standards Board (1997)</td>
<td>Unpublished document WHO/BLG/97.1</td>
</tr>
<tr>
<td>Biological products prepared by recombinant DNA technology</td>
<td>Adopted 1990, TRS 814 (1991)</td>
</tr>
<tr>
<td>Diphtheria, tetanus, pertussis (whole cell) and combined (DTwP) vaccines</td>
<td>Revised 1989, TRS 800 (1990); Addendum 2003, TRS 927 (2005); Addendum 2005, TRS 941 (2007)</td>
</tr>
<tr>
<td>DNA vaccines; quality and nonclinical safety</td>
<td>Revised 2005, TRS 941 (2007)</td>
</tr>
<tr>
<td>Haemorrhagic fever with renal syndrome (HFRS) vaccine (inactivated)</td>
<td>Adopted 1993, TRS 848 (1994)</td>
</tr>
<tr>
<td>Hepatitis B vaccine prepared from plasma</td>
<td>Revised 1987, TRS 771 (1988)</td>
</tr>
<tr>
<td>Hepatitis B vaccines made by recombinant DNA techniques</td>
<td>Adopted 1988, TRS 786 (1989); Amendment 1997, TRS 889 (1999)</td>
</tr>
<tr>
<td>Recommendations, Guidelines and other documents</td>
<td>Reference</td>
</tr>
<tr>
<td>------------------------------------------------------------------------------------------------------------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>Human interferons prepared from lymphoblastoid cells</td>
<td>Adopted 1988, TRS 786 (1989)</td>
</tr>
<tr>
<td>Influenza vaccine (inactivated)</td>
<td>Revised 2003, TRS 927 (2005)</td>
</tr>
<tr>
<td>Influenza vaccine (live)</td>
<td>Adopted 1978, TRS 638 (1979)</td>
</tr>
<tr>
<td>Measles, mumps and rubella vaccines and combined vaccine (live)</td>
<td>Adopted 1992, TRS 848 (1994); Note TRS 848 (1994)</td>
</tr>
<tr>
<td>Pneumococcal conjugate vaccines</td>
<td>Adopted 2003, TRS 927 (2005)</td>
</tr>
<tr>
<td>Rabies vaccine for human use (inactivated) produced in cell substrates or embryonated eggs</td>
<td>Revised 2005, TRS 941 (2007)</td>
</tr>
<tr>
<td>Regulation and licensing of biological products in countries with newly developing regulatory authorities</td>
<td>Adopted 1994, TRS 858 (1995)</td>
</tr>
<tr>
<td>Recommendations, Guidelines and other documents</td>
<td>Reference</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Synthetic peptide vaccines</td>
<td>Adopted 1997, TRS 889 (1999)</td>
</tr>
<tr>
<td>Thromboplastins and plasma used to control oral anticoagulant therapy</td>
<td>Revised 1997, TRS 889 (1999)</td>
</tr>
<tr>
<td>Tuberculins</td>
<td>Revised 1985, TRS 745 (1987)</td>
</tr>
<tr>
<td>Typhoid vaccine</td>
<td>Adopted 1966, TRS 361 (1967)</td>
</tr>
<tr>
<td>Yellow fever vaccine</td>
<td>Revised 1995, TRS 872 (1998)</td>
</tr>
<tr>
<td>Yellow fever vaccine, laboratories approved by WHO for the production of</td>
<td>Revised 1995, TRS 872 (1998)</td>
</tr>
<tr>
<td>Yellow fever virus, production and testing of WHO primary seed lot 213-77 and reference batch 168-73</td>
<td>TRS 745 (1987)</td>
</tr>
</tbody>
</table>
1. **Introduction**

Human plasma is a source of important medicinal products which are obtained by a combination of large-scale processing steps known as “fractionation”. It is important that these products have an appropriate quality and safety profile.

Recognizing the importance of the provision of safe blood, blood components and plasma derivatives, the 58th World Health Assembly in 2005 (WHA Resolution 58.13) (1) expressed its support for “the full implementation of well-organized, nationally coordinated and sustainable blood programmes with appropriate regulatory systems” and stressed the role of “voluntary, non-remunerated blood donors from low-risk populations”. The provision of blood, blood components and plasma derivatives from voluntary, non remunerated donors should be the aim of all countries.

The WHO requirements for the collection, processing, and quality control of blood, blood components, and plasma derivatives were published in 1994 (2). Numerous developments have taken place since that time, requiring updates of both technical and regulatory guidelines to be made available at the global level. The recently published WHO guidelines on viral inactivation and removal procedures (3) address the measures necessary to eliminate or reduce the risk from blood-borne viruses during the processing of plasma into plasma derivatives.

The present Recommendations are intended to provide guidance on the production, control and regulation of human plasma for fractionation as a source material for plasma derived medicinal products. Such information is necessary for the manufacture of safe plasma derivatives in both developed and developing countries worldwide.

The current document, by bringing together experience and information, will serve as a guide to blood establishments in their implementation of appropriate procedures for the production and control of the starting plasma material, and will facilitate the provision of safe fractionated plasma products at the national level. It is intended to assist national (medicines) regulatory authorities in establishing the supervision necessary for assessment of the quality and safety of plasma for fractionation, either prepared locally or imported, and will therefore contribute to improved quality and safety of human plasma products worldwide. Manufacturers of plasma derivatives (fractionators) may use these guidelines when discussing the quality criteria of plasma for fractionation with representatives of blood establishments and the national regulatory authority.

This guidance document addresses only human plasma sourced for the manufacture of plasma derivatives. Plasma for clinical use is not discussed, nor is there any consideration of plasma from other species.
2. **International Biological Reference Preparations**

The full list of WHO Biological Reference Preparations relevant to blood products and related substances is available at: [http://www.who.int/bloodproducts/ref_materials/](http://www.who.int/bloodproducts/ref_materials/)

The biological activity of blood products should be measured by comparison with the relevant International Standard. Activity is usually expressed in International Units (IU), but may in some cases be expressed in SI units.

3. **Glossary**

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

**Apheresis**
Procedure whereby blood is removed from the donor, separated by physical means into components and one or more of them returned to the donor.

**Blood collection**
Procedure whereby a single donation of blood is collected in an anticoagulant and/or stabilizing solution, under conditions designed to minimize microbiological contamination of the resulting donation.

**Blood component**
A constituent of blood (erythrocytes, leukocytes, platelets or plasma) that can be prepared under such conditions that it can be used either directly or after further processing for therapeutic applications.

**Blood establishment**
Any structure or body that is responsible for any aspect of the collection and testing of human blood or blood components, whatever their intended purpose, and for their processing, storage, and distribution when intended for transfusion.¹

**Donor**
A person who gives blood or plasma used for fractionation.

**Factor VIII**
Blood coagulation factor VIII, deficient in patients with haemophilia A. Also called antihaemophilic factor.

¹ A blood centre is a blood establishment.
**Factor IX**
Blood coagulation factor IX, deficient in patients with haemophilia B.

**First-time tested donor**
A person whose blood or plasma is tested for the first time for infectious disease markers in a blood establishment.

**Fractionation**
A (large-scale) process by which plasma is separated into individual protein fractions, that are further purified for medicinal use (variously referred to as plasma derivatives, fractionated plasma products or plasma-derived medicinal products). The term fractionation is used to describe a sequence of processes, including: plasma protein separation steps (typically precipitation and/or chromatography), purification steps (typically ion-exchange or affinity chromatography) and one or more steps for the inactivation or removal of blood-borne infectious agents (most specifically viruses and, possibly, prions).

**Fractionator**
A company or an organization performing plasma fractionation to manufacture plasma-derived medicinal products.

**Genome equivalents (GE)**
The amount of nucleic acid of a particular virus assessed using nucleic acid testing.

**Good Manufacturing Practice (GMP)**
That part of quality assurance which ensures that products are consistently produced and controlled to the quality standards appropriate to their intended use and as required by the marketing authorization or product specification. It is concerned with both production and quality control.

**Hepatitis A virus (HAV)**
A non-enveloped, single-stranded RNA virus, causative agent of hepatitis A.

**Hepatitis B surface antigen (HBsAg)**
The antigen on the periphery of hepatitis B virus.

**Hepatitis B virus (HBV)**
An enveloped, double-stranded DNA virus, causative agent of hepatitis B.

**Hepatitis C virus (HCV)**
An enveloped, single-stranded, RNA virus, causative agent of hepatitis C.

**Hepatitis E virus (HEV)**
A non-enveloped, single-stranded RNA virus, causative agent of hepatitis E.
**Hepatitis G virus (HGV) (or GB virus C (GBV-C))**
An enveloped single-stranded RNA virus, causative agent of hepatitis G.

**Human immunodeficiency virus (HIV)**
An enveloped, single-stranded RNA virus, causative agent of acquired immunodeficiency syndrome (AIDS).

**Incidence**
The rate of newly-acquired infection identified over a specified time period in a defined population.

**Inventory hold period**
Period during which the plasma for fractionation is on hold pending identification and elimination of possible window-phase donations.

**Intravenous immunoglobulin (IVIG)**
Also known as immune globulin, intravenous.

**Look-back**
Procedure to be followed if it is found retrospectively that a donation from a high-risk donor should have been excluded from processing.

**Manufacture**
All operations of procurement of materials (including collection of plasma for fractionation) and products; production; quality control; release; storage; distribution; and quality assurance of plasma-derived medicinal products.

**Nucleic acid testing (NAT)**
A method to detect viral genome that uses amplification techniques such as polymerase chain reaction.

**National regulatory authority**
WHO terminology for referring to national medicines regulatory authorities. Such authorities promulgate medicines regulations and enforce them.

**Plasma**
The liquid portion remaining after separation of the cellular elements from blood collected in a receptacle containing an anticoagulant, or separated by continuous filtration or centrifugation of anticoagulated blood in an apheresis procedure.

**Plasmapheresis**
Procedure in which whole blood is removed from the donor, the plasma is separated from the cellular elements and at least the red blood cells are returned to the donor.
**Plasma products**
A range of medicinal products (as listed in Appendix 1) obtained by the process of fractionation of human plasma. Also called plasma derivatives, fractionated plasma products, or plasma-derived medicinal products.

**Plasma for fractionation**
Recovered plasma or source plasma used for the production of plasma products.

**Plasma master file**
A document which provides all relevant detailed information on the characteristics of the entire human plasma used by a fractionator as starting material and/or raw material for the manufacture of subintermediate or intermediate plasma fractions, constituents of the excipient and active substance(s), which are part of a medicinal product.

**Prevalence**
The rate of infection identified, including both past and present infections, at a specified point in time or over a specified time period in a defined population.

**Prion**
The infectious particle associated with transmissible spongiform encephalopathies. It is believed to consist only of protein and to contain no nucleic acid.

**Production**
All operations involved in the preparation of plasma-derived medicinal products, from collection of blood or plasma, through processing and packaging, to its completion as a finished product.

**Recovered plasma**
Plasma recovered from a whole blood donation and used for fractionation.

**Repeat-tested donor**
A person whose blood or plasma has been tested previously in the blood establishment for infectious disease markers.

**Replacement donor**
Person who gives blood upon request of a specific patient or patient’s family or acquaintance, which in principle is intended to be used specifically for the treatment of that patient.

**SD-plasma**
Solvent/detergent-treated pooled plasma intended as a substitute for fresh frozen plasma (FFP).
**Serious adverse event**
Any untoward occurrence associated with the collection, testing, processing, storage and distribution of blood and blood components that might lead to death or life-threatening, disabling, or incapacitating conditions for patients or which results in, or prolongs, hospitalization or morbidity.

**Serious adverse reaction**
An unintended response in a donor associated with immunization that is fatal, life-threatening, disabling, incapacitating, or which results in, or prolongs, hospitalization or morbidity.

**Source plasma**
Plasma obtained by plasmapheresis for further fractionation into plasma products.

**Traceability**
Ability to trace each individual unit of blood or blood component derived thereof from the donor to its final destination, whether this is a recipient, one or more batches of medicinal product or disposal. The term is used to describe both forward tracing (donation to disposition) and reverse tracing (disposition to donation).

**TT virus (TTV)**
A non-enveloped, single-stranded DNA virus, causing post-transfusion hepatitis of unknown etiology.

**Viral inactivation**
A process of enhancing viral safety in which the virus is intentionally “killed”.

**Viral removal**
A process of enhancing viral safety by removing or separating the virus from the protein(s) of interest.

**West Nile virus (WNV)**
An enveloped single-stranded RNA virus, causative agent of West Nile fever.

### General considerations

#### 4.1 Range of products made from human blood and plasma

Human blood is the source of a range of medicinal products. Blood products obtained from the processing of single donations of blood or plasma,
generally known as blood components, include red cell concentrates, platelet concentrates, leukocyte concentrates and plasma for transfusion. Small pools, usually of less than 10 donations, mainly for the production of platelet concentrates, can also be prepared by blood establishments. Small pool cryoprecipitate is produced in some countries. The safety of these blood components depends largely on the criteria used for selection of the donors and the screening of donations.

Other blood products are obtained by the industrial processing of plasma of a large number of donations (up to tens of thousands) that are pooled together. These products include pooled virally-inactivated plasma for transfusion that is not fractionated, and the purified plasma products, also known as plasma derivatives, that are obtained by a fractionation process that combines protein purification and viral inactivation and removal steps.

Table 1 summarizes the range of products made from human blood and plasma, illustrating the diversity of source material and manufacturing methods involved, and, consequently, the complex regulation needed to ensure their quality and safety, in particular with regard to the control of risks of infection.

Plasma-derived products are regarded as medicinal products worldwide and their marketing authorization, which involves the official approval of the production process and quality assurance (QA) system used as well as of product efficacy, should be the responsibility of the national regulatory authority in all Member States. The national regulatory authority has the duty to enforce regulations, to evaluate the quality and safety of products, and to conduct regular assessment and inspection of the manufacturing sites.

An important part of the evaluation of the marketing authorization for plasma products relates to the production and control of the starting plasma used for fractionation, and is the focus of these Guidelines.

4.2 Composition of human plasma

Human plasma is a complex biological material composed of hundreds of biochemical entities, some of which have not yet been fully characterized. Among these are albumin, various classes of immunoglobulins, coagulation factors, anticoagulants, protease inhibitors, and growth factors. The complexity of plasma is illustrated in the Table 2.

The concentrations of the various protein components vary from about 40 g/litre (albumin) down to a few nanograms/ml for some coagulation factors. Plasma protein molecular mass varies from several million daltons...
(the von Willebrand multimer complex) to tens of thousands Daltons (for example, albumin).

Table 1
Range of blood/plasma products derived from single donor or pooled donations

<table>
<thead>
<tr>
<th>Single-donor blood components</th>
</tr>
</thead>
<tbody>
<tr>
<td>■ Whole blood</td>
</tr>
<tr>
<td>■ Red cell concentrate</td>
</tr>
<tr>
<td>■ Platelet concentrate (obtained by apheresis)</td>
</tr>
<tr>
<td>■ Leukocyte concentrate</td>
</tr>
<tr>
<td>■ Plasma for transfusion</td>
</tr>
<tr>
<td>■ Cryoprecipitate</td>
</tr>
<tr>
<td>■ Cryo-poor plasma</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Small-pool blood components</th>
</tr>
</thead>
</table>
| ■ Platelet concentrates (obtained from whole blood)
| ■ Cryoprecipitate

<table>
<thead>
<tr>
<th>Large-pool, unfractionated virally inactivated plasma product</th>
</tr>
</thead>
<tbody>
<tr>
<td>■ Plasma for transfusion, solvent-detergent (SD) treated (4)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Large-pool products purified by fractionation of plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>■ See the list of products in Appendix 1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>a Usually 4–10 platelet concentrates derived either from platelet-rich-plasma or from buffy coats.</td>
</tr>
<tr>
<td>b Rarely produced. Pooled cryoprecipitate should ideally be subjected to a viral inactivation treatment. Also used as a fibrinogen source for fibrin sealant (fibrin glue).</td>
</tr>
</tbody>
</table>

Human plasma for fractionation is the starting material for the manufacture of a range of medicinal products used for the treatment of a variety of life-threatening injuries and diseases. A list which includes the most established clinical use of these products is provided in Appendix 1.

4.3 Pathogens present in blood and plasma

A number of infectious agents can be present in human blood but not all blood-borne pathogens can be transmitted by plasma for transfusion or by plasma derivatives (7). Some pathogens are exclusively associated with blood cells, or are at least partially sensitive to the freeze–thaw process that takes place during the manufacture of plasma and plasma products. In addition, the multiple sterilizing filtration steps systematically included in the manufacture of plasma products, as for any other parenteral preparation, eliminate micro-organisms larger than 0.2 µm. Table 3 summarizes the major infectious risks linked to blood-borne pathogens and presents the current evidence on risks of infection from cellular components, plasma and fractionated plasma products.
Table 2

**Selected proteins of human plasma**

<table>
<thead>
<tr>
<th>Major proteins</th>
<th>Daltons</th>
<th>mg/litre</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>• Albumin</strong></td>
<td>68 000</td>
<td>40 000</td>
</tr>
<tr>
<td><strong>• IgG</strong></td>
<td>150 000</td>
<td>12 500</td>
</tr>
<tr>
<td><strong>Protease inhibitors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>• Alpha-2-macroglobulin</strong></td>
<td>815 000</td>
<td>2 600</td>
</tr>
<tr>
<td><strong>• Alpha-1-antitrypsin</strong></td>
<td>52 000</td>
<td>1 500</td>
</tr>
<tr>
<td><strong>• C1-esterase inhibitor</strong></td>
<td>104 000</td>
<td>170</td>
</tr>
<tr>
<td><strong>• Antithrombin</strong></td>
<td>58 000</td>
<td>100</td>
</tr>
<tr>
<td><strong>• Heparin cofactor II</strong></td>
<td>65 000</td>
<td>100</td>
</tr>
<tr>
<td><strong>• Alpha-2-antiplasmin</strong></td>
<td>69 000</td>
<td>70</td>
</tr>
<tr>
<td><strong>Protease</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>• ADAMTS13</strong></td>
<td>190</td>
<td>1</td>
</tr>
<tr>
<td><strong>Fibrinolytic proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>• Plasminogen</strong></td>
<td>92 000</td>
<td>200</td>
</tr>
<tr>
<td><strong>• Histidine-rich glycoprotein</strong></td>
<td>75 000</td>
<td>100</td>
</tr>
<tr>
<td><strong>Coagulation factors and anti-coagulant proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>• Fibrinogen</strong></td>
<td>340 000</td>
<td>3 000</td>
</tr>
<tr>
<td><strong>• Fibronectin</strong></td>
<td>250 000</td>
<td>300</td>
</tr>
<tr>
<td><strong>• Prothrombin</strong></td>
<td>72 000</td>
<td>150</td>
</tr>
<tr>
<td><strong>• Factor XIII</strong></td>
<td>320 000</td>
<td>30</td>
</tr>
<tr>
<td><strong>• Protein S</strong></td>
<td>69 000</td>
<td>29</td>
</tr>
<tr>
<td><strong>• Von Willebrand Factor (monomer)</strong></td>
<td>220 000</td>
<td>10</td>
</tr>
<tr>
<td><strong>• Factor IIa</strong></td>
<td>72 000</td>
<td>150</td>
</tr>
<tr>
<td><strong>• Factor X</strong></td>
<td>59 000</td>
<td>10</td>
</tr>
<tr>
<td><strong>• Factor V</strong></td>
<td>286 000</td>
<td>7</td>
</tr>
<tr>
<td><strong>• Factor XI</strong></td>
<td>80 000</td>
<td>5</td>
</tr>
<tr>
<td><strong>• Factor IX</strong></td>
<td>57 000</td>
<td>5</td>
</tr>
<tr>
<td><strong>• Factor XII</strong></td>
<td>76 000</td>
<td>40</td>
</tr>
<tr>
<td><strong>• Protein C</strong></td>
<td>57 000</td>
<td>4</td>
</tr>
<tr>
<td><strong>• Factor VII</strong></td>
<td>50 000</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>• Factor VIII</strong></td>
<td>330 000</td>
<td>0.3</td>
</tr>
<tr>
<td><strong>Cytokines</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>• Interleukin-2</strong></td>
<td>15 000</td>
<td>Traces</td>
</tr>
<tr>
<td><strong>• Granulocyte colony-stimulating factor (G-CSF)</strong></td>
<td>20 000</td>
<td>&lt; 30 pg/ml</td>
</tr>
<tr>
<td><strong>• Erythropoietin</strong></td>
<td>34 000</td>
<td>0.3 µg/litre</td>
</tr>
</tbody>
</table>

Source: Adapted from references 5 and 6.

a Factor II is the zymogen plasma protein which upon activation generates thrombin, one of the components of fibrin sealant (fibrin glue).

b There are several cytokines present in traces in plasma. G-CSF and erythropoietin for therapeutic use are obtained by recombinant technology.

Some of the viruses listed in Table 3 are highly pathogenic (e.g. HIV, HCV and HBV), others are pathogenic only in certain recipient populations (e.g. cytomegalovirus (CMV) and B19) and a few are currently considered to be non-pathogenic (HGV and TTV).

Historically, clinical use of single-donor blood components and pooled plasma products (plasma derivatives) has been associated with transmission of blood-borne viruses (HBV, HCV, HIV, HAV and B19) (3). The
implementation of validated virus inactivation and removal steps into the manufacturing process of plasma derivatives has now virtually eliminated the risks of infection from HIV, HBV, and HCV (3) and has also avoided the transmission of some emerging infectious agents, such as WNV (8, 9).

The infective agents for the bacterial and parasitic infections most commonly associated with transfusions of cellular blood components are reliably removed during the processing and aseptic filtration of plasma products, as are residual blood cells.

### Table 3

<table>
<thead>
<tr>
<th>Infectious agents</th>
<th>Cellular blood Components</th>
<th>Plasma</th>
<th>Plasma products</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Viruses</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIV I and II</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HBV</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HCV</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hepatitis Delta virus</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HAV</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HEV</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HGV</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TT virus</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Parvovirus B19</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Human T-cell leukaemia virus I and II</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Cyto megalovirus</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Epstein–Barr virus</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>West Nile virus</td>
<td>+</td>
<td>?</td>
<td>–</td>
</tr>
<tr>
<td>Dengue virus</td>
<td>+</td>
<td>?</td>
<td>–</td>
</tr>
<tr>
<td>Human herpes virus-8</td>
<td>?</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Simian foamy virus</td>
<td>?</td>
<td>?</td>
<td>–</td>
</tr>
<tr>
<td>Severe acute respiratory syndrome (SARS) virus</td>
<td>?</td>
<td>?</td>
<td>–</td>
</tr>
<tr>
<td><strong>Bacteria</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spirochaete (syphilis)</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Parasites</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Babesia microti (babesiosis)</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Plasmodium falciparum (malaria)</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Leishmania (Leishmaniasis)</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Trypanosoma cruzi (Chagas Disease)</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Unconventional agents /TSE</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Creutzfeldt Jakob disease agent</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Variant Creutzfeldt Jakob disease agent</td>
<td>+</td>
<td>?</td>
<td>–</td>
</tr>
</tbody>
</table>

HIV, human immunodeficiency virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HAV, hepatitis A virus; HEV, hepatitis E virus; HGV, hepatitis G virus; TSE, transmissible spongiform encephalopathies.

+, evidence of transmission; –, no evidence of transmission; ?, questionable or unknown.

* Most viral transmissions associated with plasma products took place prior to the introduction of efficient viral inactivation or removal procedures.

b Transmitted by contact with animal blood but not reported to follow transfusion.

c Limited epidemiological surveys have not revealed transmission of SARS coronavirus by transfusion but further confirmation may be needed.

d Investigational studies performed by plasma fractionators using spiked TSE agents indicate that several purification steps used in the manufacture of some plasma products are likely to remove prion agents. These data may not necessarily be extrapolated to clearance of the endogenous form of the TSE agent in human blood.
4.4 Strategies to ensure safety of plasma products

A combination of measures to exclude infectious donations, together with steps to inactivate or remove potential contaminating viruses during processing, has significantly reduced the risk of disease transmission by plasma products.

There are four distinct complementary approaches to virus risk reduction for plasma products:

• minimizing the virus content of the plasma pool by:
  — implementing a quality system to select donors;
  — screening blood/plasma donations; and
  — performing plasma manufacturing pool testing.
• inactivating and removing residual viruses during plasma fractionation and processing (3);
• adherence to GMP at all steps of the production; and
• recognizing and responding appropriately to post-donation events affecting plasma donations that have already been processed.

In-process and finished product virus inactivation and/or removal procedures have been shown to play a powerful role in ensuring the viral safety of plasma products, in particular from HIV, HBV, and HCV risks (3). Those procedures have also recently been shown to provide a sufficient margin of safety against emerging lipid-enveloped viruses, such as WNV (8, 9).

Although procedures for the inactivation and removal of viruses may therefore seem to offer the fractionator an ideal means for counterbalancing occasional lapses in the identification of risk donations, such an assumption would be incorrect. As powerful as the contribution of properly validated and implemented steps for virus inactivation and removal has been shown to be, it remains essential to limit the virus load at the stage of the plasma pool by avoiding, through donor selection and donation screenings, the inclusion of a high-titre infectious donation. The synergistic effects of reduced viral load in the plasma pool and validated viral inactivation and removal procedures are well illustrated for resistant non-enveloped viruses, such as parvovirus B19, for which viral reduction procedures used during fractionation alone may not be sufficient to ensure safety (10, 11).

Exclusion of infectious donations, and retrospective identification of any infectious donation that would have passed undetected through the screening and testing nets, require the highest priority at the blood establishment. The blood establishment should establish a reliable mechanism to ensure consistent identification of such donations.

Neither of the sets of measures described above can, in isolation, provide sufficient assurance of safety against all potential blood-borne pathogens.
For this reason, the manufacture of plasma for fractionation according to Good Manufacturing Practices (GMP) is necessary to ensure the optimal quality and margin of safety of this raw material for the manufacture of medicinal plasma products.

5. **Measures to exclude infectious donations**

The safety and quality of plasma for fractionation results from the combination of several cumulative prevention measures

— appropriate selection of blood/plasma donors;
— testing of blood/plasma donations;
— epidemiological surveillance of the donor population;
— strict adherence to GMP; and
— post-donation information system.

Such information on collection and testing of plasma is requested by some regulatory authorities as part of a plasma master file (12) used in the evaluation of the marketing authorization of plasma-derived medicinal products. However, the plasma master file is not a universally used regulatory document.

5.1 **Appropriate selection of blood/plasma donors**

Plasma for fractionation should be obtained from carefully selected, healthy donors who, after review of their medical history (the donor questionnaire), medical examination and laboratory blood tests, would be considered not to present an increased risk for transmission of infectious agents by plasma-derived products (see Appendix 2). Local national regulatory authorities are pivotal in setting up at the national level a harmonized donor selection criteria framework appropriate to the country in which plasma is collected, taking into account the type of products to be manufactured, the relevant risks of infection, and the epidemiological situation. The local national regulatory authority should also be part of any decision making process intended to modify the donor selection and donation testing procedures. Specific selection criteria may be added by the plasma fractionator as part of the contractual agreement with the provider of plasma.

Regulatory agencies and a number of organizations have published regulations and recommendations concerning the criteria for the selection of donors of whole blood and of plasma obtained by apheresis (see for instance *Guide to the preparation, use and quality assurance of blood components* of the Council of Europe (13). In general these regulations and recommendations can be used as reference documents for the collection of plasma for fractionation, although some specifications may differ from those of plasma for transfusion. Examples of criteria for the selection
of donors for the collection of plasma for fractionation are presented in Appendix 2. These are not intended to constitute an absolute reference or an exhaustive list of requirements, but rather to provide examples and explain critical points for consideration.

A regular donor is someone who routinely donates blood or plasma in the same centre in accordance with the minimum time intervals. The period taken into account may vary from country to country. A repeat donor is someone who has donated before in the same establishment, but not within the period of time required to be considered as regular donation. Plasma fractionators may implement their own criteria for donors’ eligibility to improve safety margins. Whenever possible, plasma for fractionation should be collected through a donation system that relies on regular and repeat donors. Obtaining plasma from regular and repeat donors makes a major contribution to ensuring optimal historical medical information about the donors, and therefore to detecting potential risk factors.

In some countries family or replacement donors may constitute a significant proportion of the population of blood plasma donors, and — depending on the situations — have been found (14) or not (15) to be at a higher risk than regular/repeat donors of having markers of viral infections. The decision to use this plasma for fractionation is to be made jointly by the plasma fractionator and the national regulatory authorities and should be based on both a careful epidemiological assessment and the evaluation of other safety measures in place for screening of donations for viruses.

Plasma may be collected by plasmapheresis from donors who have acquired immunity through natural infection or through active immunization. Specific information on this issue can be found in Appendix 3.

5.2 Screening of blood/plasma donations for infectious markers

5.2.1 Screening tests

The following tests, considered mandatory by all regulatory agencies, are relevant to the preparation of plasma for fractionation and should be performed on each blood or plasma donation:

— an approved test for HBsAg;
— an approved test for anti-HIV; and
— an approved test for anti-HCV.

The results of all three tests should be negative. Testing for HIV p24 antigen and HCV core antigens may increase the sensitivity. Initially reactive donations should be retested in duplicate by the same assay. A repeatedly reactive donation should not be used for therapeutic applications and should usually be destroyed unless useful for non-therapeutic use.
or investigations. A sample of the donation should be evaluated by a confirmatory test and if confirmation is positive a system should exist to notify and counsel the donor. It is recommended that national algorithms should be developed and used to enable consistent resolution of discordant or unconfirmed results.

5.2.2 Other tests

The screening of plasma for fractionation for anti-human T-lymphotropic virus (HTLV) is not required as the virus is cell-associated and susceptible to inactivation by the freeze–thaw process.

In some countries, testing for anti-HBc is performed on whole blood donations as a means to reduce the risks of exposure to donations of blood components that are hepatitis B-positive (16). However, donations of plasma for fractionation obtained from whole blood that are both anti-HBc positive and HBsAg negative, and which contain a sufficient titre of antibodies against hepatitis B surface antigen (anti-HBs) are usually used for fractionation: the scientific rationale is to maintain a sufficient titre of anti-HBs antibody in the plasma pool to neutralize any HBV that may be present. The minimum titre of anti-HBs for an anti-HBc positive/HBsAg negative plasma donation to be accepted for fractionation may be specified by the plasma fractionator and/or the national regulatory authority. Currently, the minimum titre of anti-HBs antibodies required by some plasma fractionators ranges from 50 to 100 IU/l. Alternatively, the plasma donation may be identified by the plasma collector as being anti-HBc positive and the plasma fractionator may conduct additional tests. The setting of a minimum limit, if any, for the titre of anti-HBs antibody usually involves a risk assessment that takes into consideration the sensitivity of the HBsAg screening test, the testing or not of HBV by nucleic acid testing (NAT), and the efficiency of the viral reduction techniques (3, 17).

Additional testing for other agents or markers may be required by the national regulatory authority, taking into consideration the epidemiological situation in any given area or country, or the frequency of donating blood or plasma, and at the specific request of the plasma fractionator.

5.2.3 Nucleic acid testing

NAT of plasma for fractionation may be performed for the following viruses: HCV, HBV, HIV, HAV, and/or B19. If NAT is performed by the fractionator, following current practice using mini-pool samples, a specific logistics system may have to be developed at the blood establishments to collect and provide labelled samples in a form suitable for the test. In addition to performing mini-pool testing, fractionators re-test the plasma manufacturing pool for the absence of various viral markers.
5.2.4 Test kits

A system should exist in the country or region for approval of tests kits, such as an official approval system by the national regulatory agency or a delegated laboratory. The required sensitivity of the tests for the different antigens or antibodies should be determined by the national regulatory authority. In addition, the test kits to be used should be agreed by the fractionator that will receive the plasma for fractionation.

5.2.5 Quality control of screening

The quality of the screening of blood/plasma donations relies on a number of measures, such as:

— validation of new techniques before implementation;
— internal control of reagents and techniques on a daily basis;
— confirmation of positive tests by an appropriate laboratory; and
— external proficiency testing which involves the testing of a panel of sera circulated to laboratories by an approved reference institution.

Details on sampling, test equipment, validation of performance of assays, test interpretation and downloading and follow-up of reactives can be found in section 7 on QA and GMP in these guidelines.

5.2.6 Look-back

A system should be in place to perform a look-back procedure, preferably using a computer database. A look-back is a procedure to be followed if it is found retrospectively that a donation should have been excluded from processing, e.g. because that unit was collected from a donor who was subsequently rejected because of reactive viral marker, risk behaviour, exposure to CJD/vCJD or other risks related to infectious diseases. The blood establishment should then transmit this information to the fractionator according to the agreements in place, and to the national regulatory authority. Donor notification and counselling are recommended both for purposes of donor health and for the safety of the blood supply.

5.3 Epidemiological surveillance of donor population

To ensure optimal long-term safety of plasma for fractionation, it is highly recommended to establish continuous epidemiological surveillance of the donor population. This is not a requirement in all regions of the world. The objective of this survey is to know, as precisely as possible, the prevalence and incidence, and their trends, of infectious markers that are relevant to the safety of medicinal plasma products so that counter-measures can be taken in a timely fashion.
The system should not only be able to gather epidemiological data at the national and regional level but also among the donor populations that are providing blood or plasma for fractionation at individual blood establishments within a country or a region.

The information from the epidemiological surveillance can furthermore be used:

- to detect differences among donor populations of various collection centres which may be associated with objective differences in viral markers within donor populations or may reflect differences in the process of donor selection and screening among collection centres;
- to detect trends in infectious markers which may reflect either a change in the rate of viral markers in the population or a possible deviation in the donor selection or screening process at specific collection sites;
- to assess the relevance of any prevention measures such as a strengthened donor selection process, additional exclusion criteria, or implementation of additional screening tests to avoid contamination of plasma products.

When donations from first-time donors are used to prepare plasma for fractionation, epidemiological data on this specific group of donors should be included in the estimation of the risk for infectious diseases transmitted by blood. Indeed, it has been shown that first-time donors, who may occasionally include test-seeking individuals, constitute a group which in some situations is more likely to have blood-borne viral markers than regular donors group who have already gone through a selection or deferral process (18–21). Some plasma fractionators do not fractionate plasma from first-time donors as prevalence of infectious diseases may be higher in this donor group. Currently, it is advisable to collect and analyse epidemiological data at the collection sites for anti-HIV-1 and anti-HIV-2, anti-HCV and HBsAg, since, historically, they represent the major pathogenic risks associated with plasma products. It is the responsibility of the local national regulatory authority to determine whether the list should be modified or should include additional criteria, such as emerging infectious agents, based on local or regional epidemiology. For the three currently recommended markers, only confirmed positive tests (i.e. tests that are repeatedly reactive in a screening test and positive in at least one confirmatory test) should be recorded. When the plasma fractionator performs additional tests (such as NAT tests) on donations which gave negative results in serological tests, the results should be reported.

Recent guidelines published by the European Medicines Agency (EMEA) entitled *Guideline on epidemiological data on blood transmissible infections* (22) describe how to conduct epidemiological surveillance of the donor population.
5.4 **Strict adherence to good manufacturing practices**

Because the pooling of thousands of plasma donations is required for the manufacture of plasma derived medicinal products, it is necessary to ensure full traceability between individual blood/plasma units collected and the final plasma products manufactured. This is important to enable any quality and safety problems, in particular problems related to infectious risks, to be traced back to individual blood/plasma donations and to allow relevant measures to be taken to protect the donors as well as the patients who received the plasma-derived medicinal products.

The donor selection process, the collection of blood or plasma and the processing of the donation, in order to obtain plasma for fractionation, represent the first steps in the manufacturing of plasma-derived medicinal products, and therefore should be performed in compliance with GMP. Strict adherence to the principles of GMP and the implementation of a QA system to address and comply with the requirements of GMP is crucial at all stages of the production of plasma for fractionation (See section 7 on QA and GMP in these Guidelines).

5.5 **Post-donation events**

There should be a system to ensure effective communication between the blood establishment and the fractionator so that information on significant post-donation events may be immediately transmitted to the fractionator and the national regulatory authority. In particular, this procedure should allow early and effective communication of any evidence for the presence of blood-transmissible infection in a donor whose plasma has been sent for fractionation.

6. **Production of plasma for fractionation**

6.1 **Methods used to obtain plasma for fractionation**

Human plasma for fractionation may be obtained by separation of plasma from whole blood, or by apheresis.

6.1.1 **Recovered plasma**

Recovered plasma is plasma recovered by centrifugal separation from the cells and cellular debris of whole blood under the conditions described below.

6.1.2 **Apheresis plasma (source plasma)**

Apheresis plasma is obtained by a procedure in which anticoagulant-treated blood is removed from the donor, the plasma is separated from the formed
elements, and at a minimum the red cells are returned to the donor. The separation of cellular elements and plasma may be achieved either by centrifugation or filtration. The equipment used for the collection of plasma by automated methods is designed for separating cellular elements and plasma by centrifugation or filtration. The manufacturers of the equipment provide operating manuals that include instructions for installation validation, routine preventive maintenance, periodic performance checks (e.g. weight scale checks), alert mechanisms (e.g. haemoglobin detector) and troubleshooting. Annual preventive maintenance should be performed by a qualified field service engineer. It includes e.g. visual inspection, initial operational integrity, inspection of equipment integrity, inspection of filter and/or centrifuge, calibration testing and safety testing. In addition, the manufacturers of the equipment usually provide support for the installation and train on-site technicians to maintain the equipment. Apheresis collection potentially increases the availability of plasma for fractionation, enabling higher donation frequency and a larger volume per donation, and is the preferred approach for the regular collection of plasma from hyperimmune donors who have high antibody titres against specific disorders.

In principle, the method of preparation should remove cells and cell debris as completely as possible and should be designed to prevent the introduction of microorganisms. No antibacterial or antifungal agent is added to the plasma. The residual blood cell content of the plasma, in the absence of dedicated leukoreduction filtration, may vary with the collection method.

6.2 Characteristics of plasma for fractionation

6.2.1 Plasma frozen within 24 hours of collection

Subject to appropriate handling (storage and transport), plasma frozen, at −20 °C or −30 °C, within 24 hours of blood collection or apheresis (see section 6.6.2.1) will normally be suitable for optimal recovery of both labile factors (factor VIII and other coagulation factors and inhibitors) and stable plasma proteins (usually albumin and immunoglobulins). Plasma meeting these quality specifications is also used for direct clinical applications; it is then referred to as fresh frozen plasma (FFP), clinical plasma or plasma for transfusion. Table 4 sets out the main characteristics of plasma prepared either from whole blood (recovered plasma) or by apheresis.

Both sources of plasma have been found by experience to be appropriate for the manufacture of the whole range of plasma products. That said, the method of collection and preparation has some impact on the characteristics and/or yield of the proteins fractionated from the plasma. Apheresis plasma collected from donors undergoing frequent plasmapheresis contains lower levels of IgG than plasma units produced by moderate serial plasmapheresis or from whole blood (23, 24). The content of various coagulation factors is
usually higher in apheresis plasma than in recovered plasma \((24, 25)\), for various reasons that include rapid separation of blood cells and plasma, differing ratios of anticoagulant added, and the possibility of freezing the apheresis plasma soon after completion of collection.

Table 4

**Characteristics of plasma for fractionation used in the manufacture of labile plasma products**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Recovered plasma</th>
<th>Apheresis plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume, ml</td>
<td>100–260(^a)</td>
<td>450–880(^b)</td>
</tr>
<tr>
<td>Protein content, g/l (each donation)</td>
<td>≥ 50 (13)</td>
<td>≥ 50</td>
</tr>
<tr>
<td>Factor VIII, IU/ml (average)</td>
<td>≥ 0.7 (26)</td>
<td>≥ 0.7</td>
</tr>
<tr>
<td>Concentration of anticoagulant</td>
<td>Variable, according to donation size (volume of anticoagulant is fixed for a given pack type; the acceptable blood volume range should be specified)</td>
<td>Constant (metered into donation)</td>
</tr>
<tr>
<td>Acceptable donation frequency</td>
<td>Determined nationally, usually a maximum of one donation every 2 months</td>
<td>Determined nationally</td>
</tr>
</tbody>
</table>

\(^a\) Based on a standard donation size of 450 ml, with blood:anticoagulant ratio of 7:1. The maximum volume of blood to be collected during one donation procedure is determined by national authorities.

\(^b\) With anticoagulant. The maximum volume of plasma to be removed during one plasmapheresis procedure is determined by national authorities.

Preservation of factor VIII and other labile factors depends on the collection procedure and on the subsequent handling of the blood and plasma. With good practice, an average of 0.7 IU/ml factor VIII can usually be achieved both with apheresis and recovered plasma. Units of plasma for fractionation with a lower activity may still be suitable for use in the production of coagulation factor concentrates, although the final product yield may be reduced.

The implementation of GMP in the preparation of plasma for fractionation should ensure that the plasma bioburden is controlled, labile proteins are conserved as far as possible, and minimal proteolytic activity is generated.

6.2.2 **Plasma frozen after 24 hours of collection**

Plasma may be available that does not fulfil the above-defined criteria but still has value as a source of some plasma proteins. This would include:

- plasma separated from whole blood and frozen more than 24 h but usually less than 72 h after collection;
- plasma, separated from whole blood stored at 4 °C, and frozen within 72 h of separation but within the assigned shelf-life of the blood;
• Plasma frozen within 24 h but stored under conditions that preclude its use for the manufacture of coagulation factors.

Provided the circumstances of manufacture and storage of such plasma does not result in increased bioburden, the plasma may be considered suitable for the manufacture of stable plasma proteins, but not coagulation factors.

Plasma which is not frozen within 72 h of collection or separation from whole blood should not be used for fractionation.

6.2.3 Plasma not meeting the requirement for fractionation

Plasma obtained by therapeutic plasma exchange does not meet the criteria for fractionation into plasma products. Indeed, plasma from individuals subjected to therapeutic plasma exchange for the treatment of a disease state may present an enhanced risk of transmitting blood-borne diseases (due to infectious risks associated with plasma) and a high risk of irregular antibodies, and should not be offered for fractionation. In addition, such plasma cannot be classified as being obtained from a voluntary donor.

Plasma from autologous blood donations is excluded from use as plasma for fractionation and may have higher prevalence of viral markers (27).

6.2.4 Hyper-immune (antibody-specific) plasma

Detailed information regarding immunization of donors for the preparation of hyperimmune plasma is provided in Appendix 3. The following are the three approaches for the preparation of plasma for the manufacture of specific immunoglobulins (antibody-specific immunoglobulins):

• Individuals selected from the normal population by screening of plasma units for antibody titres (Screening may be random, or may be informed by knowledge of history of recovery from an infectious disease — for example varicella).
• Individuals with a high titre of a specific antibody resulting from prophylactic immunization.
• Volunteers recruited to a panel for a targeted immunization programme.

The clinical and ethical requirements for such a programme are considered in Appendix 3.

Clinically relevant specific immunoglobulins include anti-D (anti-Rho), and HAV, HBs, tetanus, varicella/herpes zoster and rabies immunoglobulins. Hyperimmune globulins are prepared for intramuscular administration, but products for intravenous use are also available. The typical derivation of hyperimmune plasma of each specificity is summarized in Table 5.
Acceptable minimum antibody potencies in individual plasma donations for fractionation should be agreed to by the fractionator. Those will usually depend upon:

- the size and composition of the fractionation pool (which may include high-titre donations to increase the mean titre of the fractionation pool);
- the characteristics of the immunoglobulin fractionation process; and
- the minimum approved potency of the final IgG product.

The following general guidance may be useful for each specificity.

### 6.2.4.1 Anti-D (anti-Rho)

- Antibody potency should be estimated in international units, using an appropriate quantitative assay (e.g. auto analyser-based assay or flow cytometry method) agreed by the fractionator.

### 6.2.4.2 Anti-hepatitis A

- Antibody potency should be estimated in international units, using a quantitative assay agreed by the fractionator.
- The minimum acceptable potency in an individual donation is unlikely to be less than 50 IU/ml.

### 6.2.4.3 Anti-hepatitis B

- Antibody potency should be estimated in international units, using a quantitative assay that detects antibody to hepatitis B surface antigen (typically radioimmunoassay (RIA) or enzyme-linked immunoassay (ELISA)) agreed to by the fractionator.
- The minimum acceptable potency in an individual donation is unlikely to be less than 10 IU/ml.

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Natural immunity</th>
<th>Prophylactic immunization</th>
<th>Targeted immunization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-D (anti-Rho)</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Anti-hepatitis A (anti-HAV)</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Anti-hepatitis B (anti-HBs)</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Anti-tetanus</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Anti-varicella/herpes zoster</td>
<td>Yes</td>
<td>No</td>
<td>Possibly</td>
</tr>
<tr>
<td>Anti-cytomegalovirus (anti-CMV)</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Anti-rabies</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>
6.2.4.4 **Anti-tetanus**

- Antibody potency should be estimated using either a neutralization assay or a quantitative assay with established correlation to the neutralization assay, agreed by the fractionator.

6.2.4.5 **Anti-varicella/zoster**

- Antibody potency should be estimated using a quantitative assay (typically ELISA, immunofluorescence or complement fixation) agreed by the fractionator.
- The minimum potency should be shown to be equal to or greater than that of a control sample provided by the fractionator.

6.2.4.6 **Anti-cytomegalovirus**

- Antibody potency should be estimated using a quantitative assay (typically ELISA, immunofluorescence or complement fixation) agreed by the fractionator.
- The minimum potency should be shown to be equal to or greater than that of a control sample provided by the fractionator.

6.2.4.7 **Anti-rabies**

- Assessing plasma for rabies antibody is rarely done. A donor may be considered to have acceptable antibody titres between 1 and 3 months after a second (or booster) dose of vaccine. Plasma should not be collected from persons immunized after exposure to infection with rabies virus.

6.3 **Premises and devices for collection of plasma for fractionation**

6.3.1 **Premises**

The collection of blood or plasma for fractionation should be performed in licensed, or regulated, permanent premises or mobile sites which are compliant with the intended activity and comply with the GMP standards approved by the national regulatory authority. The area for blood donors should be separated from all processing and storage areas. The area for donor selection should allow confidential personal interviews with due regard for the safety of donors and personnel. Before premises are accepted for mobile donor sessions, their suitability should be assessed against the following criteria:

- the size (to allow proper operation and ensure donor privacy);
- safety for staff and donors; and
- adequate ventilation, electrical supply, lighting, hand washing facilities, blood storage and transport equipment, and reliable communication capabilities.
6.3.2 Containers

Because plasma is a complex and variable mix of proteins in aqueous solution, the way in which it is handled will have consequences for its safety, quality and quantity. Furthermore, the effects of mishandling will not always be as simple (or as obvious) as a reduction in the content of recoverable factor VIII — they are just as likely to affect the behaviour of the plasma when it is thawed (this is very important to the fractionator, who requires consistency from this particularly important process step).

The containers used for the collection and storage of plasma for fractionation should comply with the appropriate regulatory provisions and should be under the control of the regulatory authority. Containers should also comply with the regulatory and technical requirements of the plasma fractionator. Containers should be labelled with batch numbers traceable to individual donations. The quality of containers has a direct impact on the quality of the plasma produced and it is therefore part of GMP to control the suitability of this starting material before use.

Containers for whole blood collections are the same as for donations of whole blood from which plasma is used for fractionation. They should be plastic, and should have been manufactured in such a way as to assure internal sterility; they should be hermetically sealed to exclude contamination. If the container is not manufactured as an integral part of a blood collection set, there should be a mechanism for docking with the collection set that minimizes the risk of adventitious microbial contamination.

Validation studies will be required to confirm the suitability of the container material (and the material of any tubing or harness through which plasma should pass) during storage in contact with the plasma. Specifically, it will be necessary to establish that the plastic is physically compatible with the proposed methods for freezing and opening (or thawing) the packs and to establish the quantities of extractable materials (for example, plasticizers) during the claimed periods of storage in the liquid and frozen forms. These studies are carried out by the manufacturer of the containers. When using collection sets and containers previously established by a manufacturer as being suitable, a cross-reference to such a study may be sufficient. Validated collection and storage containers for blood/plasma are available from several manufacturers worldwide.

The choice of the containers (e.g. type of plastic bags for recovered plasma or plastic bags or bottles for apheresis collection) has a direct impact on the design of the container opening machine that is used at the plasma fractionation plant at the plasma pooling stage.

6.3.3 Anticoagulants

Most anticoagulant solutions developed and introduced for the collection of blood cellular components and plasma for transfusion are compatible with
the preparation of plasma for fractionation and with the manufacture of plasma products (although some influence on factor VIII content in plasma has been described (28–32)). One exception is when heparin is added to the anticoagulant solution. The main anticoagulant solutions currently in use for collection of either whole blood or apheresis plasma are listed in Table 6.

Anticoagulant solutions should comply with the appropriate regulatory provisions. They can be already present in the collection container (e.g. plastic containers used for whole blood collection) or added to the blood flow during apheresis procedures. In both cases, information on the device and the anticoagulant should be provided to the regulatory authorities. The fractionator will need to know what anticoagulant has been used, and its concentration as these may have an impact on the fractionation process.

6.4 Blood/plasma collection process

6.4.1 Procedure

A standardized and validated procedure for the preparation of the phlebotomy site should be followed using a suitable antiseptic solution, and should be allowed to dry (depending on the type of disinfectant). The prepared area should not be touched before insertion of the needle. Prior to venipuncture the containers should be inspected for defects. Any abnormal moisture or discoloration suggests a defect. A careful check of the identity of the donor should be performed immediately before venipuncture.

The collection of a whole blood unit used to prepare plasma for fractionation should be performed following already established recommendations (for instance as described in the Council of Europe Guide (13)). In particular, good mixing of the blood with the anticoagulant solution should be ensured as soon as the collection process starts to avoid risks of activation of the coagulation cascade. The mixing can be done manually, every 30 to 45 seconds, and at least every 90 seconds. Collection of one standard unit of blood should be achieved within 15 minutes, as longer collection periods may result in activation of the coagulation factors and cellular components.

In automated apheresis procedures, whole blood is collected from the donor, mixed with anticoagulant, and passed through an automated cell separator. The plasma for fractionation is separated from the cellular components of the blood, which are returned to the donor in a series of collection/separation and return cycles. The plasma is separated from the red blood cells by centrifugation or filtration, or a combination of both (33, 34). The operational parameters of the plasmapheresis equipment are defined by the manufacturers of the equipment and by the requirements of national regulatory authorities. In general, the anticoagulant (often 4% sodium citrate)
is delivered at a rate to yield a specified ratio of anticoagulant to blood. The volume of plasma collected from the donor during one procedure and over a period of time is regulated. The number of collection/separation and return cycles for each donor depends on the total volume of plasma that is to be collected. For determining the number of cycles employed, the equipment requires programming by input of data. These data elements may include such parameters as donor weight and haematocrit values. The amount of time required for the donation procedure depends on the number of cycles (and hence the volume of plasma collected) but is generally between 35 and 70 minutes.

Table 6
Examples of anticoagulant solutions commonly used in the preparation of plasma for fractionation

<table>
<thead>
<tr>
<th>Composition</th>
<th>Recovered plasma</th>
<th>Ratio per 100ml blood</th>
<th>Apheresis plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACD-A</td>
<td></td>
<td></td>
<td>(x)</td>
</tr>
<tr>
<td>Sodium citrate dihydrate 22.0 g/l</td>
<td>×</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Citric acid hydrous 8.0 g/l</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dextrose monohydrate 25.38 g/l</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH (25 °C) 4.7–5.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACD-B</td>
<td></td>
<td></td>
<td>25</td>
</tr>
<tr>
<td>Sodium citrate dihydrate 13.2 g/l</td>
<td>×</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citric acid hydrous 8.0 g/l</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dextrose monohydrate 15.18 g/l</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH (25 °C) 4.7–5.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPD</td>
<td></td>
<td></td>
<td>(x)</td>
</tr>
<tr>
<td>Sodium citrate dihydrate 26.3 g/l</td>
<td>×</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citric acid hydrous 3.7 g/l</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dextrose monohydrate 25.5 g/l</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium biphosphate 2.22 g/l</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium hydroxide 1 N (pH adjustment)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH (25 °C) 5.3–5.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPD-A</td>
<td></td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>Sodium citrate dihydrate 26.3 g/l</td>
<td>×</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citric acid hydrous 2.99 g/l</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dextrose monohydrate 29 g/l</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium biphosphate 2.22 g/l</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenine 0.27 g/l</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium hydroxide 1 N (pH adjustment)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH (25 °C) 5.3–5.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP2D</td>
<td></td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>Sodium citrate dihydrate 26.3 g/l</td>
<td>×</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citric acid hydrous 3.7 g/l</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dextrose monohydrate 50.95 g/l</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium biphosphate 2.22 g/l</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium hydroxide 1 N (pH adjustment)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH (25 °C) 5.3–5.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4% Citrate</td>
<td></td>
<td></td>
<td>6.25</td>
</tr>
<tr>
<td>Sodium citrate dihydrate 40 g/l</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citric acid hydrous:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>as required for pH adjustment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH (25 °C) 6.4–7.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(×), seldom used; ×, commonly used.
6.4.2 *Labelling of collection bags*

There should be a secure system for procurement, printing and storing of the bar code labels used to identify the main collection bags and the satellite bags, associated samples and documentation to ensure full traceability at each stage of plasma production. There should be a defined procedure for labelling collection bags and samples — in particular to ensure that the labels correctly identify the association between samples and donations. Labelling should be performed in a secure manner, e.g. at the donor couch, prior to collection, or immediately after the start of collection, to avoid mislabelling. Duplicate number sets of bar code donation numbers should not be used. Information on the label of the donation should include: official name of the product; volume or weight; unique donor identification; name of the blood establishment; shelf-life or shelf term; shelf temperature; and name, content and volume of anticoagulant.

6.4.3 *Equipment*

Equipment used for the collection and further separation of blood should be maintained and calibrated regularly, and the collection and separation process needs to be validated. When validating the quality of the recovered plasma, a set of quality control tests, including measurement of total proteins, residual blood cells, haemoglobin, and relevant coagulation factors, such as Factor VIII, should be included. In addition, markers of activation of the coagulation and fibrinolytic systems may, if necessary, be performed with the support of the plasma fractionator based on the specifications of the plasma for fractionation set out by the fractionator and/or the national regulatory authority.

Likewise, apheresis equipment and apheresis procedures should be validated, maintained and serviced. Validation criteria for assessing the quality of plasma for fractionation also include protein recovery, residual content of blood cell and haemoglobin, and relevant coagulation factors. Validation studies of new apheresis procedures should also evaluate possible risks of activation of the coagulation, fibrinolysis, and complement systems potentially induced by the material in contact with blood (25, 35, 36); such studies are usually performed by the manufacturer of the apheresis machines.

6.4.4 *Laboratory samples*

Laboratory samples should be taken at the time of blood/plasma collection. Procedures should be designed to avoid any mix-up of samples and samples awaiting testing should be stored at an appropriate temperature, as specified in the operating instructions of the test kits.

6.4.5 *Volume of plasma per unit*

The volume of recovered plasma per container varies depending upon the volume of whole blood collected, the respective haematocrit of the donor, and
the volume of the anticoagulant solution. The volume of apheresis plasma per container depends directly upon the volume collected during the apheresis session and the volume of anticoagulant. The range of volume of blood and plasma collected per donor is usually defined in national regulations taking into consideration criteria such as the weight of the donor.

Although in most countries the volume of whole blood collected is close to 400–450 ml per donor, in some it may be as low as 200 ml (under those circumstances, the volume of anticoagulant solution is reduced so that the plasma:anticoagulant ratio is constant). As a result the volume of recovered plasma per unit (including anticoagulant) may vary from about 100 to 260 ml per container. In the case of plasmapheresis plasma, the volume may range from about 450 to 880 ml per container, depending upon the regulations in the country of collection.

The volume of plasma per container has direct practical impact on the fractionation process and manufacture of plasma products. Small-volume donations (e.g. 100 ml) will require more handling by the plasma fractionation operators at the stage of plasma preparation, at the container opening step, and during plasma thawing. The overall container opening process will take longer, requiring additional care to control bacterial contamination. Another consequence is that the number of donations contributing to a plasma pool will be higher (for instance, 20 000 plasma donations for a pool size of 2000 litres).

6.4.6 Secure holding and reconciliation

When the collection process is finished, it should be ensured that blood/plasma donations are held at the donation site using a secure system to avoid mishandling.

Prior to dispatching the collected donations to the blood/plasma processing site, reconciliation of the collected donations should be performed according to a standardized procedure. The procedure should also specify the actions to be taken if there are found to be missing numbers or leaking containers. Documentation should accompany the donations to the plasma processing site, to account for all donations in the consignment.

6.4.7 Donor call-back system

A system should be in place in the blood establishment which allows recall of a donor if further analysis or investigation is necessary.

6.5 Separation of plasma

6.5.1 Premises

Blood processing should be carried out in adequate facilities suitable for the needs of the intended activity. The donor area and plasma processing areas
should be separated whenever possible. Each area used for processing and storage should be secured against the entry or intervention of unauthorized persons and should be used only for the intended purpose. Laboratory areas and plasma storage areas should be separate from the donor and processing areas.

6.5.2 Intermediate storage and transport

Transport of the donations and samples to the processing site should be done according to procedures that ensure both constant approved temperature and secure confinement. This is especially important when blood/plasma is transported from distant blood drive sessions.

Temperature monitoring is important to ensure optimal compliance and quality. One way is to use packaging methods that can keep the blood/plasma within the required temperature limits. Portable temperature loggers can be used to monitor and record temperatures during the transportation of blood/plasma to the processing site.

6.5.3 Impact of whole-blood holding period

It has been shown that whole blood anticoagulated with CPD, transported and stored at 22 °C for up to 8 h prior to separation of plasma is suitable for the production of plasma for fractionation, but factor VIII activity is reduced by an additional 15–20% if blood is stored for 24 h (37). Rapid cooling of whole blood to 22 °C +/– 2 °C immediately after collection (e.g. using cooling units with butane-1,4-diol) (38) protects factor VIII and may allow storage of blood for 24 h (39). A temperature of 4 °C during transportation or storage of blood collected with either ACD, ACD-adenine, or CPD anticoagulants consistently appears to reduce the factor VIII content, but not necessarily that of other proteins, especially after 8 hrs of holding time (40–43). Holding blood at 4 °C for longer than 8 h is therefore not recommended when plasma is used for fractionation in the manufacture of factor VIII products.

6.5.4 Centrifugation of whole blood

Documentation on blood and plasma collection should be checked at the processing laboratory on receipt of the donations; reconciliation between consignment and documentation received should be performed. Blood separation procedures should be performed using a closed system and should be validated, documented and proven to ensure that containers are correctly identified.

Reproducible production characteristics of the plasma for fractionation, following a validated procedure, should ensure consistency in the residual blood cell count and protein content and quality to meet the specifications
set out by the blood establishment or the national regulatory authority and the plasma fractionator.

Comparisons have shown that CPD whole blood units that were centrifuged under conditions of low g force for a long time and those subjected to high g force for a short time yielded blood components of similar quality (44). Blood separation classically starts with the isolation of the platelet-rich plasma (PRP) fraction from whole blood by low-speed centrifugation. Subsequent high-speed centrifugation of PRP in turn yields the corresponding platelet concentrate and the plasma.

Fully automated systems for blood processing including removal of the buffy-coat layer have replaced manual extraction procedures. This allows standardized extraction and contributes to compliance with GMP in the preparation of blood components including plasma for fractionation (45). Blood component separation systems may be based on buffy coat extraction by the “top and bottom” technique (46). Its efficacy in terms of yield, purity, and standardization of blood components has been well established.

Several technical approaches have been developed to separate blood components. The process may involve normal centrifugation to separate the blood components, which are subsequently squeezed out from the top and bottom simultaneously under control of a photocell. This primary separation step results in three components: a leukocyte-poor red-cell suspension, plasma, and a buffy-coat preparation (46). A multiple-bag system with top and bottom drainage of the primary bag allows automatic separation of blood components; plasma containing $14.6 \pm 5.6 \times 10^3$ platelets/µl and $0.04 \pm 0.035.6 \times 10^3$ leukocytes/µl is obtained (47). Blood components may be separated by initial high-speed centrifugation ($4158$ g, 14 min, $22 ^\circ C$) of whole blood in sealed triple or quadruple bag systems, followed by simultaneous extraction of fresh plasma at the top, and the red blood cell concentrate at the bottom, of the respective satellite bags that constitute the blood extraction bag system — keeping the leukocyte-platelet buffy coat layer stable throughout the process within the original extraction bag. The buffy coat component yields the platelet concentrate after low-speed centrifugation and removal of the plasma from the PRP. Automatic separators that subsequently express the various components into their respective satellite bags in top and bottom systems yield plasma containing $3 \pm 3 \times 10^6$ leukocytes and $4 \pm 3 \times 10^8$ platelets per unit (48). The “top and bottom” approach allows a marked reduction in leukocyte contamination of the different blood components (38, 49), and may yield optimal plasma volume (38).

6.5.5 Impact of leukoreduction

Recently, several countries have implemented universal leukoreduction of the blood supply (50, 51) to avoid cell-mediated adverse events or improve viral
safety of blood components. It has also been considered as a precautionary measure against the risk of transmission of variant Creutzfeldt-Jakob disease (vCJD). A recent study in an endogenous animal infectivity model reports that leukoreduction of whole blood removes 42% of the vCJD infectivity associated with plasma (52), whereas further investigation by the same group found a ~70% removal of infectivity (R. Rohwer, unpublished data). The impact of leukoreduction on plasma protein recovery and activation markers appears to depend upon the chemical nature of the filters (53, 54). Some loss of coagulation factors and sometimes an increase in the markers of coagulation and complement activation has been found, although the impact on the quality of fractionated plasma derivatives is not known (54, 55). Therefore, until more scientific data are gathered, the benefits of leukoreduction for the quality and safety of plasma products remains debated. The decision to leukoreduce plasma for fractionation should be assessed with the plasma fractionator and the national regulatory authority.

6.6 Freezing of plasma

Freezing is an important processing step that has an impact on some aspects of the quality of plasma for fractionation, in particular with regard to the content of factor VIII.

Several aspects of the freezing conditions of plasma for fractionation have been evaluated.

6.6.1 Holding time of plasma

Holding plasma, freshly harvested from CPD-whole blood, at ~ 4 °C for up to 24 h before freezing at –20 °C for 4 months was shown to induce almost 25% loss of factor VIII activity compared to that in plasma frozen immediately, whereas other coagulation factors were not affected (56). Storing plasma at 22 °C for 2–4 h does not seem to induce a significant loss of factor VIII activity; however, after 4 h, some loss of activity takes place (41, 57).

Therefore, placing recovered plasma in a freezer as soon as possible, or at least within 4 h, after separation from cellular elements, would be favourable to the recovery of factor VIII. Similarly apheresis plasma should be frozen as soon as possible after completion of the collection procedure.

6.6.2 Freezing rate and freezing temperature

6.6.2.1 Freezing conditions

The regulatory requirements for the temperature at which plasma should be frozen vary (58), and depend upon the type of proteins fractionated.

The fractionator may also wish to specify freezing conditions depending on the intended use of the plasma.
The European pharmacopoeia currently states that recovered or apheresis plasma for fractionation to be used for the manufacturing of labile proteins (e.g. production of factor VIII concentrate) should be frozen rapidly, within 24 hours of collection, at –30 °C or colder (26), as this temperature has long been claimed to ensure complete solidification (59), and to be needed for optimal freezing (60). However, freezing conditions are currently under debate and the wording used in the European pharmacopoeia monograph may be revised. Recovered plasma used to manufacture only stable plasma proteins (e.g. albumin and immunoglobulins) should be frozen within 72 h of collection at –20 °C or colder.

The US Code of Federal Regulations specify that plasma collected by apheresis and intended as source material for further manufacturing should be stored at –20 °C or colder immediately after collection.

The rate at which freezing proceeds is considered to be an important quality factor, at least when coagulation factors are intended to be produced (61, 62). Rapid freezing of plasma prevents or reduces loss of factor VIII in frozen plasma either recovered or obtained by apheresis (23, 63, 64), whereas slow freezing of plasma has been shown to influence the purity and recovery of factor VIII in cryoprecipitate (61, 64–66). An ice front velocity of 26 mm/h during freezing was recently shown to preserve factor VIII:C in plasma better than 9 mm/h or less (57).

Therefore, freezing plasma rapidly (typically in less than 2 h, so as to ensure a high ice front velocity) down to a core temperature of at least –20 °C, and preferably colder, appears to be the best approach for the preservation of labile proteins.

6.6.2.2 Impact of containers and equipment

To ensure optimal and consistent freezing and storage conditions, it is important to use standardized plasma containers as freezing time is influenced by container shape, volume and thickness (57, 64, 65).

Optimum conditions used by some plasma collectors to ensure reproducible freezing are achieved by freezing “well separated” plasma packs in a stream of moving cold air at the lowest temperature tolerable to the plastic of the pack (a so-called “blast freezer”), and then to store the frozen packs “close-packed” in a storage freezer at the agreed upon storage temperature. The worst case would be to place a large number of unfrozen plasma bags, close together, in a domestic (–18 °C to –22 °C) freezer, adding more plasma bags for freezing each day, and storing the plasma under these conditions for several months. With good practice at the time of loading (i.e. not putting too many packs in at the same time and keeping them separated), a walk-in freezer at a suitable temperature offers a workable compromise.
The plasma fractionator should specify precisely to the plasma collector, with the approval of the national regulatory authority, which precise freezing parameters to use.

6.6.2.3 **Validation of the freezing process**

Recovered plasma and apheresis plasma should be shown to be frozen in a consistent manner at the required temperature. A system should be in place for ensuring that plasma is frozen to the correct core temperature within the time limit agreed upon with the plasma fractionator, keeping in mind that the speed of freezing will be influenced by the type of plasma container as well as by the volume of plasma (64). Validation of the freezing process by recording the temperature of plasma donations during a freezing process allows evaluating the freezing capacity of the equipment to be evaluated. Validation studies should be available, and should demonstrate that the temperature of a frozen pack reaches the proposed storage temperature following the specifications agreed upon with the manufacturer.

As indicated above, the aim should be to achieve rapid freezing, and thereafter to minimize temperature changes to the frozen plasma.

6.7 **Storage of plasma**

6.7.1 **Storage conditions and validation**

Plasma for fractionation should be stored at –20 °C or colder.

A multicentre study showed no detectable storage-related changes in three pools of plasma (2 recovered CPD plasma and 1 apheresis plasma) that had been quick-frozen at –30 °C, or colder, and stored over a period of 36 months at –20 °C, -25 °C, -30 °C, or –40 °C. An 11% reduction in factor IX was found in one of the recovered plasma pools during storage at –20 °C for 2 years (67). The authors concluded that plasma may be stored at –20 °C for 2 years, or at –25 °C, –30 °C, or –40 °C for 3 years.

By keeping the average storage temperature of the frozen plasma as constant as possible, at or below –20 °C, the original quality of the plasma is maintained, without having any impact on the fractionation process, in particular the cryoprecipitation step (60, 61, 66).

The *European pharmacopoeia* has a provision stating that if the temperature of the plasma is between –20 °C and –15 °C for a maximum of 72 h, or if it is above –15 °C (but colder than –5 °C) in no more than one occurrence, the plasma can still be used for fractionation. Therefore, maintaining a constant storage temperature of –20 °C or colder is a recommended approach to ensure a consistent and optimal plasma quality.
6.7.2 **Premises and equipment**

Storage conditions should be controlled, monitored and checked. Temperature records should be available to prove that the full plasma containment is stored at the temperature agreed upon with the plasma fractionator throughout the storage area. Appropriate alarms should be present and regularly checked; the checks should be recorded. Appropriate actions on alarms should be defined. Areas for storage should be secured against the entry of unauthorised persons and should be used only for the intended purpose. Storage areas should provide effective segregation of quarantined and released materials or components. There should be a separate area for rejected components and material.

If a temporary breakdown of the freezing machine or failure of the electricity supply occurs (e.g. electricity used for the stored plasma), examination of the temperature records should be made together with the plasma fractionator to evaluate the impact on plasma quality.

6.7.3 **Segregation procedures**

The following should be taken into account in the storage and boxing of plasma for fractionation.

- Untested plasma and released plasma should be stored in separate freezers, or if both types of plasma are stored in a single freezer a secure segregation system should be used.
- Initially reactive plasma donations should be stored in a separate quarantine freezer or a secure system (e.g. validated computer hold system) should be used to prevent boxing of non-released plasma.
- Donations that are found to be unacceptable for fractionation should be retrieved, disinfected and discarded using a secure system.
- Plasma donations for shipment to the plasma fractionator should be boxed in a secure manner and an effective procedure (such as a computerized system) should exist to make sure that only fully tested and released plasma donations are boxed.
- Prior to shipment, plasma boxes should be reconciled appropriately.
- Prior to release of the plasma shipment to the fractionator, there should be a formal review of the documentation to ensure that the plasma shipped complies fully with the specifications agreed upon with the plasma fractionator.

The goal of the above-mentioned measures is to make sure that donations that do not comply with the specifications agreed upon with the fractionator will not be released and shipped, and that traceability of donations is ensured.

6.8 **Compliance with plasma fractionator requirements**

Any plasma collected and prepared for fractionation should meet the plasma product manufacturer requirements as the specifications of plasma
for fractionation are part of the marketing authorization granted by the national regulatory authority for a specific plasma derivative. In addition, to the regulatory criteria related to donor selection and screening of donations, the quality specifications agreed upon with the fractionator may encompass:

— compliance with GMP during production and control;
— residual level of blood cells (platelets, leukocytes) that should be below a certain level that may vary depending upon the requirements of different countries or fractionators;
— protein content possibly including a minimal mean level of Factor VIII coagulation activity if this product is manufactured;
— guarantee of an appropriate ratio of plasma:anticoagulant solution (see Table 6) and evidence of appropriate mixing with the anticoagulant during the collection process (for instance, clots should be absent);
— acceptable maximum titre of ABO blood group antibodies (risks of haemolytic reactions due to the presence of ABO antibodies, or antibodies to other blood group systems, in intravenous IgG and low-purity factor VIII preparations have been described (68)). The European pharmacopoeia requires an ABO titre of less than 1:64 for the release of plasma products for intravenous use.
— maximum haemoglobin content;
— absence of haemolysis;
— colour;
— absence of opalescence (due to lipids);
— citrate (anticoagulant) range content (usually between 15 and 25 mM); and
— minimum titre of a specific antibody when the donation is used for the production of hyperimmune IgG such as anti-Rho, anti-HBs, anti-tetanus or anti-rabies.

6.9 Release of plasma for fractionation

Each blood establishment should be able to demonstrate that each unit of plasma has been formally approved for release by an authorized person preferably assisted by validated information technology (IT)-systems. The specifications for release of plasma for fractionation should be defined, validated, documented and approved by quality assurance and the fractionator.

There should be a system of administrative and physical quarantine for plasma units to ensure that they cannot be released until all mandatory requirements have been satisfied. In the absence of a computerized system for control of product status, the label of the plasma unit should identify the product status and should clearly distinguish released from non-released
(quarantined) plasma. Records should demonstrate that before a plasma unit is released, all current declaration forms, relevant medical records and test results have been verified by an authorized person.

Before final product release, if plasma has been prepared from a donor who has donated on previous occasions, a comparison with previous records should be made to ensure that current records accurately reflect the donor history.

In the event that the final product is not released due to potential impact on plasma quality or safety, all other implicated components from the same donation should be identified. A check should be made to ensure that (if relevant) other components from the same donation(s) and plasma units or other components prepared from previous donations from the same donor(s) are identified. There should be an immediate update of the donor record(s) to ensure that the donor(s) cannot make a further donation, if appropriate.

6.9.1 **Plasma release using electronic information systems**

Special documented evidence is needed if release of plasma is subject to use of electronic information systems (EIS) to ensure that the system correctly releases plasma units only if all requirements are met. The following points should be checked:

- The EIS should be validated to be fully secure against the possibility of plasma which does not fulfil all test or donor selection criteria, being released.
- The manual entry of critical data, such as laboratory test results, should require independent verification by a second authorized person.
- There should be a hierarchy of personnel permitted access to enter, amend, read or print data. Methods of preventing unauthorized entry should be in place, such as personal identity codes or passwords which are changed regularly.
- The EIS should block the release of plasma or other blood components considered not to be acceptable for release. There should also be a means to block the release of any future donation from a donor.

6.10 **Packaging of plasma**

The packaging requirements should be specified by the fractionator. The specification should include the following information:

- how the plasma containers are to be packed to prevent damage during shipment;
- that plasma of different types should be kept discrete and packaged into separate cartons; and
— that each carton should have a unique identification number or a bar code which should be clearly displayed on the carton and recorded in the shipping documentation.

6.11 Transportation of plasma

Although it is possible to think of transport as an extension of storage, some additional qualification is appropriate. This need arises because of the additional requirements for risk management during transport. Plasma is at increased risk when:

- Responsibilities for storage and transportation conditions change (especially when handling is the responsibility of individuals with little understanding of the consequences of temperature elevation, as will often be the case with contract shippers).
- Plasma is moved from one freezer or container to another (especially if this involves even temporary exposure to ambient temperatures, as on the loading dock of a blood establishment or a fractionation facility).
- The usual provisions for backup in the event of failure of the refrigeration system are not available (as during sea-transportation of several weeks duration).

The recommendations for cold chain maintenance, as mentioned for plasma storage, should also apply during transportation of plasma. The arrangements for temperature control and monitoring during shipping should be clearly defined and documented. The requirements for number and location of temperature logging devices during shipping should be based on a documented assessment of risk throughout the process. The temperature to be maintained during transportation should be defined by the fractionator in accordance with relevant regulations.

The responsibilities of organizations and individuals during shipping should be identified; in particular any requirements for documented hand-over checks should be specified. The final responsibility for acceptance of quality as compliant with specification lies with the quality department of the fractionation facility.

Table 7 summarizes some recommendations on the handling of blood and plasma to optimize the recovery of labile proteins such as factor VIII in plasma. These recommendations should be examined keeping in mind that the relationship between the content of factor VIII in the starting plasma and its recovery in factor VIII concentrates is unclear (40, 69), possibly in part due to the loss of factor VIII that takes place during industrial cryoprecipitation (70) as well as during purification and virus reduction procedures.
Table 7
Processing of plasma for fractionation to optimize factor VIII stability

<table>
<thead>
<tr>
<th>Steps</th>
<th>Recommendations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole blood storage before</td>
<td>• Up to 18 to 20 h at 22 °C ± 1 °C</td>
</tr>
<tr>
<td>plasma separation</td>
<td>• Not more than 8 h at 4 °C</td>
</tr>
<tr>
<td>Freezing</td>
<td>• As soon as possible, within 24 hrs of blood collection or apheresis procedurea</td>
</tr>
<tr>
<td>Freezing rate and temperature</td>
<td>• As specified by plasma fractionator, following relevant regulations pertaining to the countries where plasma will be fractionated and products will be marketed</td>
</tr>
<tr>
<td></td>
<td>• &lt; –20 °C or colder</td>
</tr>
<tr>
<td>Storage temperature</td>
<td>• –20 °C or colder, constant</td>
</tr>
<tr>
<td>Transportation temperature</td>
<td>• –20 °C or colder, constant</td>
</tr>
</tbody>
</table>

a Collection of plasma by apheresis makes it possible to freeze plasma immediately after the end of the collection procedure by contrast to whole blood processing.

6.12 Recall system

In the case of known or suspected quality defects of a plasma unit that has already been shipped, a person within the blood establishment should be nominated to assess the need for product recall and to initiate and coordinate the necessary actions. An effective recall procedure should be in place, including a description of the responsibilities and actions to be taken. Actions should be taken within predefined periods of time and should include tracing all relevant components of the donation and, where applicable, should include look-back procedures.

7. Quality assurance system and Good Manufacturing Practices

Human plasma for fractionation is the single most critical raw material in the manufacture of plasma derivatives. Fractionators should only use plasma for fractionation from blood establishments that are subject to inspection and approved by a national regulatory authority. When the mandatory safety testing is outsourced, the laboratories need to be inspected and approved. The safety and quality of plasma for fractionation should be assured by implementation of standards at the blood establishment where plasma is prepared. These standards should be assured by implementation, at the blood establishment, of an effective QA system based on the principles of GMP.
The QA system should ensure that all critical processes such as the purchase of raw materials, starting materials, selection of donors, collection of blood and plasma, production of plasma, storage, laboratory testing, dispatch and associated quality control measures, are specified in appropriate instructions and are performed in accordance with the principles of GMP and comply with the appropriate regulations. The management should review the system regularly to verify the effectiveness and introduce corrective measures if deemed necessary.

Because the quality standards implemented at the blood establishment have such a profound impact on the quality of plasma, it is a requirement that their implementation be agreed between the blood establishment and the fractionator, under the terms of the contract for plasma supply (Appendix 5). Medicines regulatory authorities will verify that such a contract is in place and that it complies with the regulations in force.

A blood establishment should establish and maintain an active and operational quality assurance system covering all activities, and taking into account the principles of GMP. The following items are of special relevance as part of a QA system for the production of plasma for fractionation (71).

7.1 **Organization and personnel**

There should be an organization chart showing the hierarchical structure of the blood establishment and clear delineation of lines of responsibilities. All personnel should have appropriate qualifications and experience to enable them to perform their tasks and should be provided with initial and continued training. Only persons authorized by defined procedures and documented as such should be involved in the production and control of plasma. The tasks and responsibilities should be clearly documented and understood. All personnel should have clear, documented and up to date job descriptions.

Training programmes appropriate to the specific tasks of staff members should be in place, and should include at least:

— relevant principles of plasma production and plasma characteristics;
— quality assurance and GMP; and
— relevant knowledge of microbiology and hygiene.

Training should be documented and training records should be maintained. The contents of training programmes should be periodically assessed.

If certain tasks, such as separation of blood or viral safety testing, are performed externally, these should be subject to a specific written contract. The contract should ensure that the contract acceptor meets good practice requirements in all disciplines relevant to the contract giver’s activity.
7.2 **Documentation system**

Every activity that may affect the quality of the blood and/or blood component should be documented and recorded. The documentation should be designed to ensure that the work performed is standardized and that there is traceability of all steps in the process. The documentation should allow all steps and all data to be checked. All documentation should be traceable and reliable. A document control procedure should be established for review, revision history and archiving of documents. It should include a distribution list. All changes to documents should be acted upon promptly and should be reviewed, dated and signed by an authorized person. Documentation procedures should be designed, developed, validated and personnel trained in a consistent manner.

7.3 **Premises and equipment**

Premises should be located, constructed, adapted and maintained to suit the operations to be carried out. Premises should be designed to permit effective cleaning and maintenance to minimize risk of contamination. The tasks in each area should take place in a logical sequence to minimize the risk of errors.

All critical equipment should be designed, validated and maintained to suit its intended purpose and should not present any hazard to donors or operators. Maintenance, cleaning and calibration should be performed regularly and recorded. Instructions for use, maintenance, service, cleaning and sanitation should be available. There should be procedures for each type of equipment, detailing the action to be taken when malfunctions or failures occur. New and repaired equipment should meet qualification requirements when installed and authorized before use. Qualification results should be documented.

7.4 **Materials**

Only reagents and materials from approved suppliers that meet the documented requirements and specifications should be used. Where relevant, materials, reagents and equipment should meet the requirements of other local legislation for medical devices. Appropriate checks on goods received should be performed to confirm that they meet specifications. Inventory records should be kept for traceability. Critical materials should be released under the responsibility of quality assurance function before use.

7.5 **Validation programme**

All processes and equipment involved in the production and control of plasma for fractionation should be validated. Data should be available to ensure that the final product will be able to meet specifications.
7.6 **Quality monitoring data**

Quality control of plasma should be carried out according to a defined sampling plan taking into account different collection and production sites, modes of transport, methods of preparation and equipment used. Acceptance criteria should be based on a defined specification for each type of plasma for fractionation. These data may include monitoring of factor VIII or any other protein quality criteria determined by the plasma fractionator, and monitoring of residual cell counts (platelets, leukocytes, erythrocytes) when requested by the plasma fractionator. All quality control procedures should be validated before use.

The viral safety testing should be performed in accordance with the recommendations of the manufacturers of the reagents and test kits. The work record should identify the test(s) employed to ensure that entries, such as the calculation of results, are available for review. The results of quality control testing should be subject to periodic review.

Test results that do not satisfy the specified acceptance criteria should be clearly identified to ensure that plasma from that donation remains in quarantine and that the relevant samples are kept for further testing. Where possible the performance of the testing procedures should be regularly assessed by participation in a formal system of proficiency testing.

7.7 **Virology safety testing**

7.7.1 **Sampling**

The following are practical points to consider in ensuring that sampling is performed appropriately:

- **Sampling machine:**
  - *Automatic sampling:* Test samples should be taken automatically and the donation number should be read from the barcode. In case of failure of the automatic system, an appropriate system for manual entry of records of donations should exist, and should require double entry with digit checks;
  - *Sampler validation:* The sampling machine should be validated and a validation report should be available; and
  - *Calibration:* The sampling machine should be calibrated on schedule and records available.
- **Reconciliation:** There should be a reconciliation of the samples received at the virology laboratory versus expected.

7.7.2 **Test equipment**

The following are practical points to consider in ensuring that the equipment used for the virology testing performs appropriately:
• Sample addition. The process of addition of samples to the test plates should be automatic and should include identification of the barcode of the plates.
• Test processing. Ideally, the test processing should be automated. If addition of reagents is done manually, full documentation should be available.
• Equipment. Pipettes, incubators and other items of equipment should be fully validated and routinely calibrated and appropriate records maintained.

7.7.3 **Assay performance validation**

The objective of validation of assay performance is to make sure that the performance of the virology assays, as carried out by the entity responsible for plasma collection, is satisfactory. Points to consider include:

• Each test run should include an independent control.
• Analysis of positive controls.
• Analysis of data on non-repeatable reactives (see 7.7.5 below).
• Evidence of satisfactory participation in external proficiency schemes.

7.7.4 **Test interpretation and downloading**

The data from virology safety testing should be examined by the supervisor before being officially accepted. Accepted data should be downloaded directly to the mainframe computer, or there should be a secure system for manual download which ensures positive release of the samples. No transcription of results should be done as mistakes may be introduced.

7.7.5 **Follow-up of reactives**

The following should be given special attention:

• *Identification of initial reactives.* They should be identified using a secure system.
• *Repeat reactives.* An acceptable system should be in place to confirm repeat reactives, including sampling, labelling, testing, and entry of results.
• *Editing of repeat reactive.* A computer algorithm should edit reactive status to repeat reactive, or the editing should be performed by two staff members.
• *Deferral system.* An appropriate deferral system should exist for repeat reactives.
• *Re-entry of deferred donors.* Appropriate documentation should be in place.

7.8 **Electronic information system**

Importance should be given to the introduction of an EIS for blood establishments involved in the preparation of plasma for fractionation and
when possible linked to other establishments to facilitate and speed tracing of individual plasma donations. This will allow timely identification of the location of donations in the chain of production of plasma products.

All software, hardware and backup procedures should be validated before use and checked at least once a year to ensure reliability. The system should prevent the use of duplicate donation numbers, or else the system should be able to deal with duplication without data corruption.

Hardware and software should be protected against unauthorized use or changes (e.g. by password protection of key functions). There should be procedures for each type of software and hardware, detailing the action to be taken when malfunctions or failures occur.

A backup procedure should be in place to prevent loss of records during expected and unexpected downtime or function failures. Changes in computerized systems should be validated, applicable documentation revised and personnel trained, before the change is introduced into routine use. The EIS should be maintained in a validated state.

7.9 **Storage and transport**

Storage and distribution routines should take place in a safe and controlled way to assure product quality throughout storage and transport and to exclude identification errors of plasma units. Intermediate storage and transport should be carried out under defined conditions to ensure that set requirements are met.

7.10 **Change control system**

A formal change control system should be in place for planning, evaluating and documenting all changes that may affect the quality, traceability, availability or effect of components or safety of components, donors or patients. The potential impact of the proposed change should be evaluated. The need for additional testing and validation should be determined.

7.11 **Quality assurance auditing**

In order to monitor the implementation and compliance with the blood establishment quality management system, regular internal audits are needed. These should be conducted independently by trained and competent persons from within the organization, according to approved protocols. Inter-institutional audits should be actively promoted.

All audit results should be documented and reported to management. Appropriate corrective actions should be taken. Preventive and corrective actions should be documented and assessed for effectiveness after
implementation. In general the blood establishment should have procedures for systematic improvement. Input for this process can come from complaints, errors, inspections, audits and suggestions.

7.12 **Defect reporting system**

There should be systems in place to ensure that complaints, all types of quality defects (e.g. in blood bags or test kits), and adverse events or reactions are documented, carefully investigated for causative factors and, where necessary, followed by the implementation of corrective actions to prevent recurrence. This also applies to “near miss events”. The corrective and preventive action system should ensure that nonconformity of the product or quality problems are corrected, that recurrence of the problem is prevented, and that the plasma fractionator is notified according to the agreed procedure. The blood establishment should have methods and procedures in place to channel product or quality problems into the corrective and preventive action system.

7.13 **Quality agreement between blood establishment and fractionator**

The important elements of a blood establishment quality system with critical implications for plasma quality, will normally be addressed in a quality agreement — an addendum to the contract for plasma supply. The quality agreement should address at least the following areas of concern:

- agreement on specific donor selection criteria (with approval of the national regulatory authority);
- schedule of requirements for exclusion or acceptance of donors, including the arrangements for establishing donor identity and the provision for possibility of self-exclusion;
- arrangements for monitoring and reporting the epidemiology of the donor population;
- location of blood establishments (and of any facility to which a quality-critical function, for example donation testing, has been outsourced);
- frequency of donation and the system for ensuring that donations are not taken more frequently than allowed;
- requirements for donor screening and for donation testing, including any provision for the preparation and testing of mini-pools;
- procedure for validation and approval of relevant test reagents and kits;
- record-keeping, including the arrangements for traceability of donors and donations;
- specifications of plasma to be supplied, including any arrangements for verifying compliance with specifications and documentation of compliance;
• specifications of containers to be used for blood/plasma collection and supply;
• detailed requirements for labelling of individual plasma units (the adhesive used for the labels should not compromise the quality of the plasma products);
• arrangements for freezing, storage and shipment of plasma;
• notifiable events, including the arrangements for post-donation notification;
• procedure for review and approval of any proposal for procedural change;
• procedure and agreed frequency for audit of blood establishments by the fractionator; and
• arrangements for notifying the fractionator of a proposed regulatory inspection, its periodicity, and of the outcome of such an inspection.

7.14 Blood/plasma establishment audit and inspection

It is a requirement of GMP that the regulatory authorities and the plasma fractionator should establish the basis of confidence in the quality of critical raw materials. In the case of plasma, this is achieved by four basic provisions:

• maintenance of a list of blood establishments approved (by the fractionator and the regulatory authorities) for supply of plasma;
• agreement in a contract, or in the technical agreement to a contract of supply, of the quality arrangements made at each blood establishment approved for supply of plasma;
• regular audit of blood establishments to confirm satisfactory implementation of the quality arrangements (these audits should be reported in writing to the blood/plasma establishment and any remedial actions confirmed); and
• monitoring of the quality of plasma supplied, with trending of quality-critical parameters.

There will normally be a requirement for independent inspection and approval of each blood establishment by the relevant regulatory authority (see below). Such inspections should be provided for in any contract between the plasma supplier and the fractionator, and will normally be undertaken by the responsible authority in the country where plasma preparation is performed. Written reports of such inspections should be made available to the blood establishment and a remediation plan agreed upon. Reports of regulatory inspections and associated remediation plans should be made available to the fractionator under the terms of the contract for plasma supply.
8. **Regulatory control of plasma for fractionation**

8.1 **Role of national regulatory authority**

According to the WHO Guidelines for national regulatory authorities on quality assurance of biological products (72, 73), national regulatory authorities have the duty to ensure that available biological products, whether imported or manufactured locally, are of good quality, safe and efficacious, and should thus ensure that manufacturers adhere to approved standards of quality assurance and GMP. The responsibilities of the national regulatory authority should also include the enforcement and implementation of effective national regulations, and the setting of appropriate standards and control measures. The evaluation and control of the quality, safety and consistency of production of blood products involve the evaluation of the starting material, production processes and the test methods to characterize batches of the product. This requires specialist expertise by the national regulatory authority.

8.2 **Establishment licence and inspections**

In many countries, national regulatory authorities have implemented a control system based on licensing the establishments, inspecting them regularly, and enforcing the implementation of the legal requirements and applicable standards.

According to international GMP standards for the manufacturing of blood products, the following two main principles are important for the control of plasma as starting material:

- Quality assurance should cover all stages leading to the finished product, from collection (including donor selection) to storage, transport, processing, quality control and delivery of the finished product.
- Blood or plasma used as a source material for the manufacture of medicinal products should be collected by establishments and be tested in laboratories which are subject to inspection and approved by a national regulatory authority.

These two points in the GMP requirements summarize an important basic principle which is relevant for the manufacture of plasma derivatives and the control of plasma as starting material. Most national regulations therefore require that the establishments involved in the collection and storage of plasma as a source material (e.g. plasmapheresis centres and blood establishments) need to have an establishment licence and need to be inspected by the competent national regulatory authority. To obtain the licence the establishments have to fulfil a defined set of requirements to guarantee that the plasma collected is safe and of good quality. Since each unit collected represents a single batch, a marketing authorization for the plasma
as a “product“ is not required in all countries. Under the latter condition, a “system control", instead of a “product control", may be more appropriate. In addition to the establishment licensing system some countries have also introduced a product-specific approval system for blood components.

8.3 Impact of good manufacturing practices

The approach of implementing the principles of GMP in the production of medicinal products is not new, and it is widely acknowledged that it is essential in assuring the quality and safety of medicinal products. For blood products, GMP becomes even more important and more complex due to the biological nature of the products. Therefore, taking into account the principles of GMP and the existence of an appropriate system of quality assurance to address and implement these requirements in the manufacturing steps of blood products should be a pivotal element of the preparation of plasma for fractionation. As outlined in the previous sections, implementation of GMP in the manufacture of blood products is essential, and quality assurance and GMP should cover all stages, including the collection of plasma as starting material. The implementation and enforcement of GMP in blood establishments therefore has the following impact:

— introduces the application of quality assurance principles in all steps involved in the collection, preparation and testing of blood components;
— supports systematic application of donor selection criteria for each donation;
— reduces errors and technical problems in collection, preparation, testing, and distribution;
— contributes to the release of products which comply with safety and quality requirements;
— ensures adequate documentation and full traceability for each donation and product;
— enables continuous improvement in collection, preparation and testing of starting material;
— supports regional cooperation networks that may result in the formation of centres of competence by centralizing activities in order to reach compliance at the required level (cost-benefit for implementing quality assurance measures);
— provides suitable tools for the national regulatory authority to assess the compliance of a plasma collection centre.

An establishment licensing system for blood establishments by the competent national regulatory authorities should therefore exist. The main requirements for obtaining an establishment licence may include:

• Application of quality assurance system and GMP to all steps from donation, to preparation, storage, testing and distribution of plasma.
• Personnel directly involved in the collection, testing, processing, storage and distribution of plasma need to be appropriately qualified and provided with timely and relevant training.

• Adequate premises and equipment should be available.

• An adequate system to ensure traceability of plasma should be established; traceability should be enforced through accurate donor, donation, product and laboratory sample identification procedures, through record maintenance and use of an appropriate labelling system.

• Requirements for selection of donors, including exclusion criteria for donors with risk behaviours; provision of information to donors on risk situations and the donation in general; and the use of a questionnaire to obtain information on donor’s health.

• Requirements for testing of each donation.

• Requirements regarding traceability and documentation.

• Post-donation information system.

8.4 Inspections

In conducting regular inspections as part of the licensing procedure, enforcement of the implementation of GMP is required aiming to ensure the compliance of the blood establishments with the existing provisions. It is the responsibility of the inspector from the national regulatory authority to ensure that the manufacturers and the blood and plasma establishments adhere to the approved standards of GMP and quality assurance, including at sites where plasma is collected as starting material.

The inspections should be carried out by officials representing the competent national regulatory authority. These officials should be specialized inspectors, trained in GMP inspections, and they should be familiar with blood bank technologies and the special features of quality assurance in the collection of plasma. Inspections may follow common inspection procedures, including:

• an opening meeting;
• a blood establishment tour;
• inspection of main areas and activities;
  — donor acceptance and identification
  — donor suitability
  — collection process
  — processing and sampling
  — plasma freezing
  — testing and availability of test results
  — release of plasma units
  — storage, transportation and shipment
  — quality assurance (including self inspection and change control)
  — documentation (standard operating procedures, records, donor record files and log books)
— personnel and organization
— qualification and process validations
— error and corrective action system
— look-back information, recalls and complaints
— product quality controls

• a final meeting summarizing the inspection outcome.

A thorough inspection includes the observation of staff during performance operation and comparison with defined written procedures. In a “system control”, the inspection can be considered not only as a GMP inspection, but also as an indirect product quality assessment by checking product-specific validation and quality control data.

A written report should summarize the main aspects of the inspection including its scope, a description of the company, the deficiencies listed, specified and classified (e.g. as critical, major or minor), and a conclusion. The written report will be sent to the company. The companies are requested to notify the national regulatory authority about the specific steps which are taken or planned to correct the failures and to prevent their recurrence. If necessary, follow-up inspections should be performed e.g. to check that specific corrective actions have been implemented.

The national regulatory authority should have the authority to withdraw an establishment licence in a case where inspection results showed critical non-compliance with the requirements or product specifications.

Information on the collection and control of the starting material, human blood or plasma, and on the procedures conducted during the preparation of the final blood derived medicinal product have to be documented as part of the dossier in the marketing authorization.

In summary, the implementation of licensing and inspection systems for blood establishments has become an important tool through which the national regulatory authorities confirm the assurance of quality of plasma as starting material for fractionation. The use of international standards not only further promotes harmonization, but also facilitates regional collaboration and information exchange between the national regulatory authorities.

Authors

The drafts of these guidelines were prepared by:

Dr T. Burnouf, Human Plasma Product Services, Lille, France; Dr A. Padilla, World Health Organization, Geneva, Switzerland; Dr C. Schärer, Swissmedic, Swiss Agency for Therapeutic Products, Bern, Switzerland; Dr T. Snape, Consultant, Pickering, North Yorkshire, UK; Dr P. Strengers, International Society of Blood Transfusion, Amsterdam, the Netherlands; Professor S. Urbaniak, Regional
Transfusion Centre, Aberdeen, UK; Professor W.G. van Aken, Professor of Medicine, Amsterdam, the Netherlands.

The Drafts prepared were circulated for Consultation to Representatives of National Regulatory Authorities, National Blood Programs and to the respective WHO Regional Advisors in all the WHO Regional Offices. Plasma fractionators were consulted through their respective plasma fractionation associations or through the regulatory agencies in their countries. Both the Plasma Protein Therapeutic Association and the International Plasma Fractionation Association presented consolidated comments of their Members.

Acknowledgements are due to the following experts for their comments, advice and information given during the preparation and consultation process of these Guidelines:

Lic. M. P. Alvarez, Departamento Biológicos, CECMED, Havana, Cuba; Dr. R. S. Ajmani, Intas Pharmaceuticals Ltd, Chinubhai Centre, Ahmedabad, India; Dr D. Armstrong, Natal Bioproducts, South Africa; Dr. T. Barrowcliffe, National Institute for Biological Standards, Potters Bar, Herts, UK; Dr C. Bianco, America's Blood Centers, Washington DC, USA; Dr R. Büchel, Plasma Protein Therapeutics Association (PPTA) Source, Brussels, Belgium; Dr E. A. Burgstaler, Transfusion Medicine, Mayo Clinic, Rochester, Minnesota, USA; Mr A. Cadiz, Empresa Productora de Sueros y Hemoderivados, La Habana, Cuba; Dr F. Cardoso de Melo, Agencia Nacional de Vigilancia Sanitaria, Ministerio da Saude, Brasilia, Brazil; Dr B. Cuthbertson, Scottish National Blood Transfusion Service, Edinburgh, UK; Dr A. M. Cheraghali, Iran Blood Transfusion Organization, Tehran, Iran; Dr N. Choudhury, Prathama Blood Center, Vasna, Ahmedabad, India; Dr J. R. Cruz, Regional Advisor Laboratory and Blood Services, AMRO/PAHO, Washington, USA; Dr. F Décary, Héma-Québec, Canada; Dr N. Dhingra, World Health Organization, Geneva, Switzerland; Dr R. Dodd, American Red Cross, USA; Dr J. Epstein, Office of Blood Research and Review, FDA Center for Biologics Evaluation and Research, Bethesda, Maryland, USA; Mr T. Evers, International Plasma Fractionation Association (IPFA), Amsterdam, the Netherlands; Ms M. Farag, Egyptian Regulatory Authority, Cairo, Egypt; Professor A. Farrugia, Office of Devices, Blood and Tissues, Therapeutic Goods Administration, Woden, Australia; Dr B. Flan, Laboratoire Français du Fractionnement et des Biotechnologies, les Ulis, France; Dr J. C. Goldsmith, Office of Blood Research and Review, FDA Center for Biologics Evaluation and Research, Bethesda, Maryland, USA; Ms K. Gregory, AABB, Bethesda, MD, USA; Ms M. Gustafson, Plasma Protein Therapeutics Association, Washington, USA; Mrs T. Jivapaisarnpong, Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand; Dr H. Klein, National Institutes of Health, Clinical Research Center, Transfusion Medicine, Bethesda, MD, USA; Dr. J. Kurz, Federal Ministry of Health and Women, Medicines & Medical Devices Inspectorate, Vienna, Austria; Professor J. Löwer, Paul Ehrlich Institute, Langen, Germany; Mrs B. Mac Dowell Soares, Agencia Nacional de Vigilancia Sanitaria, Brasília, Brazil; Dr E. Al Mansoori, Drug Control Department, Ministry of Health, United Arab Emirates; Dr M. Maschio, Plan Nacional de Sangre, Buenos Aires, Argentina; Dr A. Miller, Blood National Program, Montevideo, Uruguay; Dr S. Park, Korea Food and Drug Administration, Seoul, South Korea; Professor I. Peake,
International Society on Thrombosis and Haemostasis, University of Sheffield, Sheffield, UK; Dr F. Reigel, Swissmedic, Swiss Agency for Therapeutic Products, Bern, Switzerland; Dr A. Robinson, NHS Blood and Transplant, National Health Service, UK; Mr D. Sato, Ministry of Health and Welfare, Japan; Professor E. Seifried, German Red Cross, Institute of Transfusion Medicine and Immunohaematology, Frankfurt/Main, Germany; Professor R. Seitz, Paul Ehrlich Institute, Langen, Germany; Dr G. Silvester; European Medicines Evaluation Agency, London, UK; Dr L. S. Slamet, National Agency of Drug and Food Control, Indonesia; Dr T Simon, Tricore, USA; Professor J.-H. Trouvin, Afssaps, Paris, France; Dr F. Vericat, Grifols, Barcelona, Spain; Dr E. Voets, Biological Standardization, Scientific Institute of Public Health, Federal Public Service Health, Brussels, Belgium; Professor G. N. Vyas, University of California, San Francisco, California, USA; Dr M. Weinstein, Office of Blood Research and Review, FDA Center for Biologics Evaluation and Research, Rockville, Maryland, USA; Mrs M. Wortley, Haemonetics, Braintree USA; Professor H. Yin, Biological Products, State of Food and Drug Administration, Beijing, People's Republic of China; Dr Mei-Ying Yu, Office of Blood Research and Review, FDA Center for Biologics Evaluation and Research, Rockville, Maryland, USA.

Special thanks are also due to Dr T. Burnouf for the compilation and professional follow up of the significant number of contributions received during the Consultation process. Dr T. Burnouf and Dr A. Padilla, WHO Project Leader prepared the final manuscript of these Guidelines.

References


61. **Farrugia A, Prowse C.** Studies on the procurement of blood coagulation factor VIII: effects of plasma freezing rate and storage conditions on cryoprecipitate quality. *Journal of Clinical Pathology*, 1985, **38**:433–437.


64. **Carlebjork G, Blomback M, Pihlstedt P.** Freezing of plasma and recovery of factor VIII. *Transfusion*, 1986, **26**:159–162.

65. International Forum — What are the critical factors in the production and quality control of frozen plasma intended for direct transfusion or for fractionation to provide medically needed labile coagulation factors? *Vox Sanguinis*, 1983, **44**:246–259.


70. **Foster PR.** Control of large-scale plasma thawing for recovery of cryoprecipitate factor VIII. *Vox Sanguinis*, 1982, **42**:180–189.


Appendix 1

**Plasma products and clinical applications**

<table>
<thead>
<tr>
<th>Products</th>
<th>Main indications</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Albumin</strong></td>
<td></td>
</tr>
<tr>
<td>Human serum albumin</td>
<td>Volume replacement</td>
</tr>
<tr>
<td><strong>Blood coagulation factors</strong></td>
<td></td>
</tr>
<tr>
<td>Factor VIII(^1)</td>
<td>Haemophilia A</td>
</tr>
<tr>
<td>Prothrombin complex</td>
<td>Complex liver diseases; warfarin or coumarin derivatives reversal(^2)</td>
</tr>
<tr>
<td>Factor IX</td>
<td>Haemophilia B</td>
</tr>
<tr>
<td>Factor VII</td>
<td>Factor VII deficiency</td>
</tr>
<tr>
<td>Von Willebrand factor</td>
<td>Von Willebrand factor deficiency (type 3 and severe forms of type 2)</td>
</tr>
<tr>
<td>Factor XI</td>
<td>Haemophilia C (factor XI deficiency)</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>Fibrinogen deficiency</td>
</tr>
<tr>
<td>Factor XIII</td>
<td>Factor XIII deficiency</td>
</tr>
<tr>
<td>Activated PCC</td>
<td>Haemophilia with anti-factor VIII (or factor IX) inhibitors</td>
</tr>
<tr>
<td><strong>Protease inhibitors</strong></td>
<td></td>
</tr>
<tr>
<td>Antithrombin</td>
<td>Antithrombin deficiency</td>
</tr>
<tr>
<td>Alpha 1 antitrypsin</td>
<td>Congenital deficiency of alpha 1 antitrypsin with clinically demonstrable panacinar emphysema</td>
</tr>
<tr>
<td>C1-inhibitor</td>
<td>Hereditary angioedema</td>
</tr>
<tr>
<td><strong>Anticoagulants</strong></td>
<td></td>
</tr>
<tr>
<td>Protein C</td>
<td>Protein C deficiency</td>
</tr>
<tr>
<td><strong>Fibrin sealant (fibrin glue)</strong></td>
<td>Topical haemostatic/healing/sealing agent (surgical adjunct)</td>
</tr>
</tbody>
</table>

\(^{1}\) Adapted from: Ala, F, Burnouf T, El-Nageh M. *Plasma fractionation programmes for developing economies. Technical aspects and organizational requirements*. Cairo, WHO Regional Publications, 1999 (Eastern Mediterranean Series).

\(^{2}\) Some factor VIII concentrates containing von Willebrand factor are effective for the treatment of von Willebrand disease.

\(^{3}\) Prothrombin complex contains factor II, factor VII, factor IX, and factor X. The content of factor VII may vary depending upon products.

\(^{4}\) May be used, in the absence of purified plasma products, for substitutive therapy in factor VII, factor X, or protein C deficiency. Whenever available, purified factor IX should be used to treat haemophilia B.

\(^{d}\) Product obtained by mixing a concentrate rich in fibrinogen and a concentrate rich in thrombin.
<table>
<thead>
<tr>
<th>Products</th>
<th>Main indications</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intramuscular immunoglobulins (IMIG)</strong></td>
<td></td>
</tr>
<tr>
<td>Normal (polyvalent)</td>
<td>Prevention of hepatitis A (also rubella, and other specific infections)</td>
</tr>
<tr>
<td>Hepatitis B</td>
<td>Prevention of hepatitis B</td>
</tr>
<tr>
<td>Tetanus</td>
<td>Treatment or prevention of tetanus infection</td>
</tr>
<tr>
<td>Anti-Rho (D)</td>
<td>Prevention of haemolytic disease of the newborn</td>
</tr>
<tr>
<td>Rabies</td>
<td>Prevention of rabies infection</td>
</tr>
<tr>
<td>Varicella/zoster</td>
<td>Prevention of chickenpox infection</td>
</tr>
<tr>
<td><strong>Intravenous immunoglobulins (IVIG)</strong></td>
<td></td>
</tr>
<tr>
<td>Normal (polyvalent)</td>
<td>Replacement therapy in immune deficiency states;</td>
</tr>
<tr>
<td>Cytomegalovirus (CMV)</td>
<td>Prevention of CMV infection (e.g. after bone marrow transplantation)</td>
</tr>
<tr>
<td>Hepatitis B</td>
<td>Prevention of HBV infection (e.g. liver transplant)</td>
</tr>
<tr>
<td>Rho (D)</td>
<td>Prevention of haemolytic disease of the newborn</td>
</tr>
</tbody>
</table>
Appendix 2

Donor selection

1. Preamble

Recognizing the importance of the provision of safe blood, blood components and plasma derivatives, the 58th World Health Assembly in 2005 (WHA Resolution 58.13) (1) expressed its support for “the full implementation of well-organized, nationally coordinated and sustainable blood programmes with appropriate regulatory systems” and stressed the role of “voluntary, non-remunerated blood donors from low-risk populations”. The provision of blood, blood components and plasma derivatives from voluntary, non remunerated donors should be the aim of all countries.

2. Information to donors

Candidate donors should receive an explanation, ideally both verbally and in writing, or by any other appropriate means such as a self-administered questionnaire, that answers to questions about their medical history and personal behaviour are necessary to determine whether they are eligible to donate blood or plasma. Written information can be in the form of a leaflet explaining the risks of infection associated with blood and plasma products; impact of social behaviour on risks of infection and risk factors for infection. This information is generally given by a licensed physician, or by a person under the direct supervision of a licensed physician, who should explain the exclusion criteria for donating blood and plasma. A convenient communication system should ensure that risk factors are well understood by the candidate donor.

Additionally, the donor should be asked to inform the blood centre if he or she feels unwell after the donation or if he or she forgot to mention a possible risk factor. This is of special importance for a donation used to prepare plasma for fractionation as it is important to be able to remove at-risk donations prior to the industrial pooling stage to avoid the potential need to destroy the plasma pool or the intermediates or products derived from it.
3. **Compliance with donor selection criteria**

3.1 **Positive identification of donors**

Upon presentation at the blood/plasma collection site, donors should be asked to identify themselves by stating their name, address and date of birth, and to supply proof of a permanent place of residence to establish a reliable means of contact, including, for example, a telephone number where they can be contacted after donation, if needed. Proof of identity (such as identity card, passport or driving licence) should be provided. Identification of donors should also take place immediately before venipuncture.

3.2 **Confidentiality**

The premises and setting of the blood/plasma collection centre (or the mobile collection unit) should allow for adequate confidentiality during the donor’s interview and the selection process so that the candidate donor will not avoid answering questions on his or her personal or private behaviour, which otherwise would compromise the safety of the plasma donation used for the fractionation process.

3.3 **Questionnaire and interview**

The assessment of each donor should be carried out by a suitably qualified person, trained in use of donor selection criteria and will involve an interview, a questionnaire and further direct questions if necessary. In order to obtain relevant and consistent information about the donor’s medical history and general health, it is recommended that the donor can review, complete and sign a pre-printed questionnaire (computer-assisted self-administered interviewing (CASI) is being developed in some regions), adapted to the type of donor (for instance, first-time donor versus regular donor). The questionnaire should be drafted in such a way that donors may easily identify whether they are in good health.

Candidate donors who are at risk of carrying a disease transmissible by blood/plasma derived products should be able to exclude themselves voluntarily after reading and responding to the donor information and/or the questionnaire. Such confidential self-exclusion should also be possible after the donation (e.g. by telephone).

The candidate donor should be asked to sign an informed consent to give blood/plasma in which he or she acknowledges an understanding of the moral responsibility behind the donation of blood/plasma.

3.4 **Physical examination, acceptance and deferral criteria**

3.4.1 **Physical examination**

Prior to the first donation and before subsequent blood donations and in case of plasmapheresis at regular intervals, a physical examination should
be carried out by a licensed physician or a physician substitute following an established procedure. Local national regulatory authorities, usually after consultation with the blood establishment, should determine the health criteria and the respective acceptable limits to be taken into consideration during physical examination, such as measurement of weight, blood pressure, pulse rate and temperature, or any other criteria considered to affect the safety of plasma-derived products or the donor.

3.4.2 Records and traceability

An appropriate computerized or, if not available, manual system should exist to keep records of the donors, of their medical history and health status, and to ensure efficient traceability of their donations. Such information provides historical perspective on the health status of the donors, including previous temporary deferrals (should they exist), and contributes to reinforcing the judgement as to whether the donation would present a risk to the quality and safety of plasma for fractionation.

3.4.3 Selection and exclusion criteria

The following elements have been recognized as playing a role in selecting the safest donors:

Establishment of exclusion criteria: Relevant acceptance, deferral and exclusion criteria for the donation of blood/plasma used for fractionation should be formulated by the national regulatory authority and be applicable nationwide, as national requirements. Within the scope of their role to establish and implement effective national regulations, local national regulatory authorities should enforce such criteria. Based on the characteristics of the production process used to manufacture plasma-derived products, the plasma fractionator may suggest additional or alternative exclusion criteria. For instance, in some countries, the plasma from first-time donors is not used.

Deferral: A defined list of permanent or temporary deferral criteria used for candidate donors from which the plasma would be used for fractionation, should be clearly stated, made public, and incorporated in the donor educational material. The physician performing the physical examination should be able to identify whether the donor has been previously deferred and, if so, for what reason. Examples of the major permanent deferral criteria found in international guidelines include:

- clinical or laboratory evidence of blood-borne infectious diseases, e.g. infection with HIV, HBV or HCV;
- past or present intravenous drug use.

Other exclusion criteria, either permanent or temporary, may include:

- sexual relationship between men;
- men or women who are engaged in prostitution;
• subjects with haemophilia or other clotting-factor defects, in particular if treated with clotting factors;
• sexual partners of any of the above or of someone the donor suspects may carry the above risk factors;
• jaundice within the 12 months prior to donation, as it may be a clinical sign of hepatitis A, B or C;
• transfusion with blood, blood components, or plasma products in the 12 months previous to donation, as blood transfusion is a risk factor for all blood-borne infections;
• tattooing, scarification, ear piercing, acupuncture in the 12 months prior to donation. These practices may be a vehicle for the transmission of viral diseases unless clear evidence is provided that the procedure was carried out under sterile conditions;
• a particular policy may be required with regard to the exclusion criteria for a risk factor relevant to the safety of cellular blood components although it does not create safety issues for the preparation of plasma for fractionation and plasma derived products. For instance, risk factors for HTLV infection (e.g. due to travel in countries where the prevalence is high) may be an exclusion criterion for the donation of blood components, but this virus cannot be transmitted by plasma products. It is however not advisable to introduce two screening and quality standards for products separated from a whole blood unit (e.g. red cell concentrates and plasma for fractionation) as this may in itself create a risk of mishandling and error at the blood collection centre.

3.4.4 Reinstatement
When temporary deferral criteria are applied, a specific procedure conducted by trained personnel should be in place for reinstatement of donors. Some exclusion criteria are temporary (e.g. as long as a risk factor has been identified) and can be waived once additional checks on the donor have been made, or the time period for exclusion has passed.

3.4.5 Procedures
Based on such criteria, a written procedure should be in place at the blood/plasma collection centre to control donor acceptance and deferral criteria. The procedure should comply with the requirements of the national regulatory authority and fractionator. Abnormal conditions should be referred to the physician who has the responsibility of making the final decision on the donor suitability. If the physician has any doubt about the donor’s suitability, donation should be deferred.

Reference
Appendix 3

Donor immunization and plasmapheresis for the manufacture of specific immunoglobulins

There is a need for hyperimmune plasma for the manufacture of specific immunoglobulins that are clinically valid for therapeutic and prophylactic uses.

**Donors with acquired antibodies**

Plasma may be collected by plasmapheresis from donors who have acquired immunity through natural infection or through active immunization with approved vaccines for their own protection. Donors with medically useful plasma may be identified by screening whole blood donations or by testing the plasma of convalescent patients or vaccinated individuals who have produced high-titre antibodies with the desired specificity, for example, patients recovering from varicella-zoster infection or donors who have been immunized with rabies vaccine. Unnecessary primary immunizations can be avoided by this approach. Donation of plasma following natural infection should be deferred until the potential donor is asymptomatic, and non-viraemic.

**Donors who require immunization**

To ensure a sufficient supply of life-saving immunoglobulins to treat patients, deliberate immunization of healthy volunteers may be necessary in addition to collection of plasma from convalescent patients and donors selected by screening for high levels of specific antibodies. The immunization of donors requires informed consent in writing and should take into consideration all the requirements of this Annex.

Donors should be immunized with antigens only when sufficient supplies of material of suitable quality cannot be obtained from other appropriate donors, or from donations selected by screening. Donors should be fully informed of the risk of any proposed immunization procedure, and pressure should not be brought to bear on a donor to agree to immunization. Women capable of child-bearing should not be immunized with erythrocytes or other antigens that may produce antibodies harmful to the fetus. Donors with known allergies should preferably not be recruited.

Every effort should be made to use the minimum dose of antigen and number of injections. In any immunization programme, the following should be taken into consideration as a minimum:

— the antibody assay;
— the minimum level of antibody required;
— data showing that the dose, the intervals between injections and the total dosage proposed for each antigen are appropriate; and
— the criteria for considering a prospective donor a non-responder for a given antigen.

A donor could be hyperimmunized with more than one immunizing preparation as long as the safety of the procedure of multiple immunizations is demonstrated.

Potential donors should be:

• informed by a licensed physician of the procedures, risks and possible sequelae and how to report any adverse effects, and encouraged to take part in a free discussion (which, in some countries, takes place with small groups of potential donors);
• informed that they are free to withdraw their consent at any time.

In addition, donors may also be:

• encouraged to seek advice from their family doctor, or from an independent competent counsellor, before agreeing to immunization; and
• informed that any licensed physician of their choice will be sent all the information about the proposed immunization procedure.

All vaccines used for immunizing donors should be approved by the national regulatory authority. Special care should be taken to ensure the safety of the donor when a vaccine is administered at doses or according to schedules that differ from those recommended for routine prophylactic immunization. Erythrocyte and other cellular antigens should be obtained from an establishment approved by the national regulatory authority. Donors should be observed for approximately 30 min following any immunization in order to determine whether an adverse reaction takes place. Because reactions often occur 2–3 h after immunization, donors should be advised of this possibility and instructed to contact the facility’s physician if a reaction is suspected in the first 12 h after immunization. Reactions may be local or systemic. Local reactions, which may be immediate or delayed, take the form of redness, swelling or pain at the injection site. Systemic reactions may include fever, chills, malaise, arthralgia, anorexia, shortness of breath and wheezing. An insurance system should be in place to compensate for side-effects to the donor.

Immunization with human erythrocytes

Erythrocyte donors

A donor of erythrocytes for the purposes of immunization should meet all the general health criteria for donors (see Appendix 2). Relevant measures should be taken to limit the risk of infectious diseases; these may vary from
country to country taking into consideration the relevant risks. For instance, in some countries, the donor should never have had a blood transfusion in order to reduce risks of vCJD. Prior to the first donation, the donor should be found to be negative for relevant markers, which may include the following: syphilis, HBsAg, anti-HIV, antibody to hepatitis B core antigen (anti-HBc), anti-HCV and antibodies to human T-cell lymphotropic viruses (anti-HTLV), and the serum level of aminotransferases should be within normal limits as established by the national control authority. Erythrocyte phenotyping should be done for ABO as well as for C, D, E, c and e. It is advantageous to select red cells expressing high amounts of RhD antigen, e.g. homozygous D or Rho, for immunization. Phenotyping for other clinically relevant specificities is also required, especially for Kell, Fya/Fyb, Jka/Jkb and S/s. The volume of erythrocytes drawn from a donor should not exceed 450–500 ml of whole blood in any 12 week period. Shorter intervals may induce iron deficiency and, possibly, anaemia. Erythrocytes obtained for immunization purposes should be frozen (at least for 6–12 months depending upon the sensitivity and range of the tests performed, e.g. the use of NAT) before use and the donor should be retested and shown to be negative for the above markers of infection before the stored cells are released and used for immunization. Pre-storage leukoreduction of donations is considered desirable, and NAT testing for HBV, HCV and HIV would give an additional level of safety.

Collection and storage of erythrocytes

Erythrocytes should be collected under aseptic conditions into sterile pyrogen-free containers in an appropriate proportion of an approved anticoagulant. They may then be dispensed in aliquots under aseptic conditions into single-dose sterile, pyrogen-free containers for storage. The microbiological safety of the dispensing environment should be validated. The method selected should have been shown to provide acceptable cell recovery in vitro (80%) or in vivo (70%). Erythrocytes should be washed after storage to remove the cryoprotective agent (e.g. glycerol). Adequate sterility data to support the shelf-life for stored erythrocytes should be kept on file. A test for bacterial and fungal contamination should be done on all blood dispensed in aliquots in an open system. The test should also be performed on at least one single-dose vial from each lot of whole blood that has been stored unfrozen for more than seven days. The test should be done on the eighth day after collection and again on the expiry date. Sterility tests should be performed following an approved procedure.

Erythrocyte recipients

The following additional testing of erythrocyte recipients is necessary:

- The recipient should be phenotyped for ABO, Rh, Kell Fya/Fyb, Jka/Jkb and S/s antigens before immunization. The red cell donor and the recipient
should be matched as far as possible for major blood group antigens other than RhD. Only ABO-compatible erythrocytes may be transfused. Whereas mismatching within the Rh system for C and or E is acceptable, mismatching in the Kell, Fy, Jk and S/s systems is unacceptable.

• Screening for unexpected antibodies by methods that demonstrate coating and haemolytic antibodies should use the antiglobulin method or a procedure of equivalent sensitivity.

Prospective erythrocyte recipients in whom antibody screening tests demonstrate the presence of erythrocyte antibodies (other than those deliberately stimulated through immunization by the plasmapheresis centre) should be asked whether they have ever been pregnant or had a blood transfusion, a tissue graft or an injection of erythrocytes for any reason. This history should form part of the permanent record and should identify the cause of immunization as clearly as possible. Recipients should be notified in writing of any specific antibodies they have developed after injection of erythrocytes. The plasma centre should maintain records, which should be reviewed during inspection. The immunized donor should carry a card or medical alert bracelet specifying the antibodies. These measures allow optimal care of immunized donors who may require an emergency transfusion, (e.g. following a road traffic accident) at some future time, and for whom knowledge of the antibody status, especially mixtures of antibodies, is important.

**Immunization schedules**

Erythrocytes used for immunization purposes should not be administered as part of any plasmapheresis procedure. Such immunization may be performed on the same day as plasmapheresis, but only after it and as a separate procedure.

To minimize the risk of infection to the donor, the immunization schedule should involve as few doses of erythrocytes as possible. Wherever possible, the same red cell donor should be used throughout the immunization programme of an individual plasma donor.

For primary immunization two injections of erythrocytes, each of a volume of about 2–5 ml and given 3 months apart, elicit antibody formation within three months of the second injection. Different schedules may be used for de novo immunization. It is advantageous to choose as donors of anti-D (anti-Rho) volunteers who are already immunized, because useful levels of anti-D are then usually attained within a few weeks of reimmunization with 2–5 ml of erythrocytes. About 70% of immunized volunteers eventually produce antibody levels well above 100 IU/ml. The baseline antibody titre of every recipient of erythrocytes should be established, and the antibody response, including both type and titre, should be monitored monthly to
establish the peak level of anti-D and duration of the response. The response of each recipient is individual, and additional injections of erythrocytes may be required at intervals of 2–9 months to maintain anti-D levels (I). If injections of erythrocytes are discontinued, antibody levels usually fall appreciably within 6-12 months. Erythrocytes to be used for immunization purposes should be selected, for each recipient, by a licensed physician or a suitably trained and qualified person.

Donors undergoing primary immunization who have not responded to a total of up to 150 ml erythrocytes are likely to be ‘non-responders’ and should be removed from the panel.

**Plasmapheresis schedules**
Donors should comply with the requirements for health screening and maximum plasma donation allowed by their national authorities.

**Risks to recipients**
Recipients of erythrocytes for immunization purposes may be at risk of:

— viral hepatitis (B and C) and HIV infection;
— other infectious diseases;
— HLA immunization;
— the production of unwanted erythrocyte antibodies that may complicate any future blood transfusion;
— a febrile haemolytic reaction if the antigen dose is too high;
— vCJD in countries where this is endemic.

**Record-keeping**
Records of erythrocyte donors and of the recipients of their erythrocytes should be maintained and cross-referenced and stored at least for the minimum time required for blood transfusion recipients by the national authorities.

**Reference**

Appendix 4

Contract plasma fractionation programme

The fractionation of plasma requires specialized facilities, with provision for large-scale protein separation, purification, virus inactivation and formulation, as well as for aseptic finishing and freeze-drying. The preparation of plasma-derived products should be governed by the same regulatory considerations that are applied to medicines. Manufacturers are required to obtain manufacturing licences which should cover the method of preparation and product characteristics. To obtain a licence, it is necessary to demonstrate adherence to GMP. Considerable technological, pharmaceutical and scientific expertise is required to meet these demands. Since key utilities (such as heating, ventilation and air-conditioning (HVAC), refrigeration and water for injection) should be maintained operational even when the facility is not fractionating plasma, the investment in and running costs of fractionation are substantial. The economic viability of a fractionation facility will be determined by:

• the cost of the plasma for fractionation (in particular cost-allocation of the whole blood collection system on plasma versus labile components);  
• the operating capacity of the facility; and  
• plasma availability and product demand to allow the facility to operate continuously at near to maximum capacity.

The break-even point for minimum annual plasma throughput for economic viability may vary greatly according to a set of parameters, these including plasma cost, product portfolio, adequacy of the various plasma products versus the plasma needed to cover those needs, and product yield. Therefore such projects require a careful feasibility study.

Countries which cannot justify building and operating a fractionation facility, may opt to have plasma collected locally and shipped for processing in an independent facility—so-called contract or toll fractionation. Plasma-derived products are then returned to the originating country on payment of a fee (toll). Such arrangements can work well, subject to specific provisions being made and adhered to. These include:

• commercial and quality agreements defining the responsibilities of both parties (the contract giver and the contract acceptor);  
• clearly defined requirements for plasma quality (including the arrangements for donor selection, testing and traceability);  
• provision for audit of the plasma collection centre (by the fractionator) and inspection by an appropriate regulatory body;  
• formal approval of the contract plasma fractionation activities by the regulatory authority of the fractionator;
a contractual commitment to supply agreed quantities of plasma. The annual minimal volume is dependent upon the fractionator’s overall free capacity and specific aspects of production such as plasma pool and product batch size;

agreement on the arrangements for storage and shipment of plasma, with defined provisions for monitoring and control (typically transport by sea, at −20 °C or below);

agreement on the range of products to be manufactured; and

agreement on specific aspects of plasma processing (including batch size, possible requirements for segregation of processing, agreed use or destruction of excess intermediates, expected yield and toll fees).

Plasma products made from local plasma need to receive a specific registration, even if the same products made from foreign plasma are already licensed in the country of origin.

The regulatory authorities of the country where the plasma is collected may require inspection of the fractionation centre. Table 1 summarizes the responsibilities and roles of each party.

<table>
<thead>
<tr>
<th>Task</th>
<th>Blood establishment</th>
<th>Plasma fractionator</th>
<th>Regulatory authority</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epidemiology surveillance of donor population</td>
<td>Collects and analyses the data based on results of screening tests</td>
<td>Reviews the data</td>
<td>Reviews the data</td>
</tr>
<tr>
<td>Donor selection and interview</td>
<td>Develops and implements the criteria in selection and interview of donors</td>
<td>Verifies that criteria set by national regulatory authority are met; may provide additional selection criteria</td>
<td>Sets the criteria and inspects the blood establishment</td>
</tr>
<tr>
<td>Serological testing of donation</td>
<td>Performs validated tests (or the tests may be subcontracted)</td>
<td>Agrees on the tests kits used and audits the virology laboratory</td>
<td>Approves test kits and inspects the blood establishment</td>
</tr>
<tr>
<td>Post-donation follow-up and haemovigilance</td>
<td>Informs plasma fractionator (and when appropriate the regulatory authority) when relevant information is obtained</td>
<td>Takes appropriate measures if plasma pool or product quality is compromised</td>
<td>Evaluates haemovigilance/post-donation reports with regards to product quality and safety</td>
</tr>
<tr>
<td>Task</td>
<td>Blood establishment</td>
<td>Plasma fractionator</td>
<td>Regulatory authority</td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>---------------------------------------------------------------------------------------------------------------</td>
<td>--------------------------------------------------------------------------------------------------------</td>
<td>---------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Preparation of plasma</td>
<td>Collects blood plasma, prepares, freezes, and stores the plasma, according to good manufacturing practice (GMP)</td>
<td>Sets the specifications and audits</td>
<td>Approves and inspects the blood establishment</td>
</tr>
<tr>
<td>Nucleic acid testing (NAT) (mini-pool)</td>
<td>Prepar...</td>
<td>Provides the standard operating procedure for NAT samples and performs (or sub-contracts) the validated testing</td>
<td>Approves the procedure and inspects the plasma fractionator</td>
</tr>
<tr>
<td>Fractionation methods including viral reduction</td>
<td>Applies the fractionation methods following GMPs and processes described in marketing authorization</td>
<td>Evaluates the data presented in the dossiers prepared by the fractionator, and inspects fractionation facility</td>
<td></td>
</tr>
<tr>
<td>Preparation of plasma product regulatory files</td>
<td>Prepar...</td>
<td>Prepares the files</td>
<td>Reviews and evaluates</td>
</tr>
<tr>
<td>GMP</td>
<td>Implements GMP</td>
<td>Audits the blood establishment</td>
<td>Inspects blood establishment and enforces GMP</td>
</tr>
<tr>
<td>Granting of marketing authorization</td>
<td></td>
<td></td>
<td>Grants the marketing authorization</td>
</tr>
<tr>
<td>Plasma product pharmacovigilance</td>
<td>Does pharmacovigilance studies and informs regulatory authorities and blood establishment when relevant side-effects are found</td>
<td>Evaluates pharmacovigilance reports with regards to product quality and safety</td>
<td></td>
</tr>
</tbody>
</table>

* See sections 7 and 8 of this annex.
Appendix 5

Technical points to consider in establishing plasma specifications criteria and obligations between blood establishment and plasma fractionator

The purpose of the contract is to have a “legally binding” document between the plasma supplier and the fractionator.

The following is an example of the quality control and documentation required by a plasma fractionator to acquire plasma for fractionation from a blood establishment. It is not meant to represent the only possible way to define plasma specifications criteria and obligations between a blood establishment and a plasma fractionator. Depending upon the prevalence of blood-borne diseases in a country, additional safety requirements on donor selection and testing should be considered.

General specifications

Donors
Reference should be made to local regulations pertaining to the selection, eligibility, and exclusion criteria for donors of blood or plasma used for the manufacture of blood components and plasma derivatives. Newly introduced criteria may be spelled out (such as travel restrictions related to vCJD).

Blood establishments
Reference should be made to the official legislation of blood establishments in the country of origin and to relevant legislation relating to plasma fractionation.

Donation process and plasma unit specifications
The contract should cover the following aspects of the donation process and plasma unit specifications.

• Collection process of the blood/plasma units:
  — containers, collection sets and anticoagulants with relevant registration;
  — duration of the whole blood collection (e.g. less than 15 minutes (I) for recovered plasma);
  — guarantee that blood will be mixed with the anticoagulant as soon as the collection starts, by regular manual shaking or using a validated automated method (I);
— prior to freezing, plasma is clear (light opalescence may be allowed),
yellow to — green in colour, with no sign of haemolysis or presence
of red cells (2); and
— acceptable citrate concentration range.

• **Infectious markers:**
— test kits used should be of acceptable sensitivity and be agreed with
manufacturer;
— anti-HIV 1 and 2, anti-HCV and HBsAg should be absent, and there
should be no laboratory evidence of syphilis;
— when applicable: specific handling of anti-HBc positive donations
(e.g. accepted only if anti-HBs antibody titre > 0.050 IU/ml and
HBsAg negative); and
— HCV NAT and HIV tests must be negative (i.e. when a blood
establishment organization performs NAT for HCV and HIV for
blood components).

• **Immunohaematological markers**
— anti-A and anti-B titre < 1/64 using a validated assay;
— special requirements relative to the absence of irregular antibodies.

• **Cellular content and haemoglobin**
— statistical records of blood cell contamination showing that the relevant
specifications are met. Some countries/fractionators have set specific
limits on the residual leukocyte content of plasma for fractionation;
— statistical records of haemoglobin contamination showing that the
relevant specifications are met.

• **Protein quality control**
— protein content ≥ 50 g/l after mixture with the anticoagulant;
— when plasma is used for production of factor VIII concentrate;
 minimum factor VIII content to be specified for a pool sample of a
defined number of donations

• **Other criteria**
— minimal acceptable volume of plasma per container;
— plasma freezing conditions: core temperature, time taken to freeze,
 and absence of folding to avoid a thin plasma layer that would be
 more susceptible to thawing during subsequent handling;
— maximum acceptable thickness of plasma containers;
— positioning of the donation identification label (number and bar
code);
— plasma storage temperature;
— plasma density (used to determine the volume of plasma shipped to/
 received by fractionator);
— maximum time elapsed between donation and shipment to the
fractionator.
**Standard plasma**

**Plasma types**

Plasma categories vary depending upon fractionator and local regulations. For instance, some fractionators may classify as plasma, either from whole blood or from apheresis, based on the time interval between the collection procedure and freezing.

Examples of plasma categories include:

- **Category A**: apheresis plasma frozen within 6 hours, with a factor VIII content $\geq 0.7$ IU/ml;
- **Category B**: Recovered plasma with a factor VIII content $\geq 0.7$ IU/ml, obtained from whole blood kept at 20–22 °C and frozen within 6 hours (in the absence of devices to maintain blood temperature), or frozen within 20 hours (if devices to maintain blood temperature are used);
- **Category C**: Plasma frozen within 24 hours after collection, or plasma initially categorized as A or B but containing $\leq 0.7$ IU factor VIII/ml. This plasma is used to produce immunoglobulins and albumin only.

**Hyperimmune plasma**

**Quality criteria**

Acceptable criteria include:

- protein content, factor VIII, haemoglobin: usually the same as for standard plasma;
- a minimum potency level will be set for each antibody type. Where possible, the required potency will be specified in IU per ml when assayed using an agreed method which includes an agreed reference control calibrated in IU/ml. Examples of limits are as follows:
  - anti-tetanus: 10 IU/ml;
  - anti-varicella/zoster: 10 IU/ml;
  - anti-HBs: 25 IU/ml;
- Indication of the assay procedure, procurement of standards, test laboratory and communication procedure of the data.

**Documentation**

Each blood establishment delivering plasma should have an approved organizational chart, and changes should be communicated to the plasma fractionation centre according to an agreed procedure.

Shipping documentation should include:

- dated shipping document signed by responsible person;
• certificate of origin and control of the plasma, stating for each donation
  the:
  —  collection date;
  —  carton number;
  —  results of virology and immunohaematology screening;
  —  test kits used and their batch number;
  —  signature of the director or an authorized person;
• password-protected electronic file of the plasma donations and samples
  sent, stating for each donation collection date (this needs to be agreed
  with the fractionator):
  —  carton number;
  —  results of virology and immunohaematology screening;
  —  test kits used and their batch number;
• upon request, additional information on viral screening tests and
  confirmatory assays can be provided to the fractionator;
• epidemiology data should be made available as appropriate, e.g.
  annually.

**Shipment**

Specifications relating to shipment include the following:

**Plasma donations**

• Broken plasma containers are not acceptable.
• When applicable, specifications of “pig tail” used for additional screening
  tests by the fractionator (e.g. length of 10–20 cm, attached to the plasma
  donation, and ideally, identified with the donation number).
• Specification of the plasma container identification (labels and barcode).
• Specification on potential additional samples sent with the shipment for
  additional screening tests such as NAT or for the look-back procedure.
• Statement on minimal number of plasma containers per shipping box or
  carton, and positioning.

**Containers for shipment**

**Auditing programme**

The contract should cover the following aspects of the auditing programme:

• obligation of the blood establishment to be subjected to auditing by the
  fractionator;
• routine auditing performed by the fractionator should follow an internally
  approved and regularly revised procedure with an established list of
  questions and check-points;
• special auditing performed annually/biannually based on a programme
  previously communicated to the director of the blood establishment;
• audit reports are communicated to the director of the blood establishment;
• list of reference documents (such as internal acceptance criteria for the preparation of plasma for fractionation).

**Notification obligations**

Notification obligations cover the following:

• obligation to notify the fractionator each time the safety of a previous donation may be questionable;
• obligation to notify the fractionator when:
  — a unit positive for viral markers such as HBsAg, HIV-1 and HIV-2 antibodies, HCV antibody or syphilis has been sent by mistake;
  — a deviation is subsequently discovered in any of the screening tests performed on the plasma units supplied. In this situation, the blood establishment should attempt to retest the implicated units if suitable library samples are available;
  — a regular donor is found to be positive for a marker although the previous donation was found to be negative;
  — the blood establishment is informed that a donor, previously contributing to plasma for fractionator, has developed an infectious disease potentially transmissible by plasma;
  — a donation is found to have transmitted an infectious disease, or there is strong evidence implicating a donation in disease transmission;
  — the blood establishment is informed that a donor previously contributing to plasma for fractionation: (a) has developed CJD or vCJD (in such a case the report with the pathological findings should be provided if available); (b) has risk factors for vCJD; or (c) is identified as exhibiting risk behaviour or other factors that affect the safety of the plasma;
  — the blood establishment is informed that a patient has developed post-transfusion infection following transfusion of blood component(s) obtained from a donor who has also donated one or more units of plasma for fractionation.

Notifications should provide the list of all donations made within a 6-month period prior to the last donation found to be negative. The period of time depends on local regulations and the type of disease. The fractionator may request additional data on previous donations when thought necessary.

A communication procedure must be in place indicating information that must be provided. This should include:

• name of qualified person at the fractionator to be contacted;
• reasons and description of the problem (under confidentiality clauses);
• the time period between information being known and communication to the fractionator;
• if the problem is related to an infectious disease, a list of all plasma for fractionation donations made in the defined period prior to the last donation found negative;
• name of the blood establishment, director, donation number, carton number as indicated on the electronic file sent with the shipment, date of shipment, date of notification and signature of the responsible person or his or her delegate.

References

Annex 5

WHO biosafety risk assessment and guidelines for the production and quality control of human influenza pandemic vaccines

This document provides guidance to national regulatory authorities and vaccine manufacturers on the safe production and quality control of human influenza vaccines produced in response to a threatened pandemic. The document details international biosafety expectations for both pilot-scale and large-scale vaccine production and control and is thus relevant to both development and production activities. It should be read in conjunction with the WHO Laboratory Biosafety Manual (1) and replaces the earlier WHO guidance Production of pilot lots of inactivated influenza vaccines from reassortants derived from avian influenza viruses: Interim biosafety risk assessment (2). Tests required to evaluate the safety of candidate influenza vaccine reference viruses by WHO Reference Laboratories prior to release to vaccine manufacturers are also specified in this document.

The following text is written in the form of guidelines rather than recommendations. Guidelines allow greater flexibility than recommendations with respect to expected future developments in the field. These guidelines specify steps to minimize the risk of introducing influenza virus strains with pandemic potential from a vaccine manufacturing facility into the community. If a national regulatory authority so desires, these guidelines may be adopted as definitive national requirements, or modifications may be justified and made by a national regulatory authority. It is recommended that modifications to the principles and technical specifications of these guidelines be made only on condition that the modifications ensure that the risks of introducing influenza virus to the community are no greater than as outlined in the guidelines set out below.

Summary
Introduction
Glossary
1. Scope of the risk assessment
2. Hazard identification
   2.1 Hazards associated with the type of pandemic vaccine viruses
      2.1.1 Hazards associated with the recipient virus in a reassortant strain
      2.1.2 Hazards arising from the inserted gene product in a reassortant vaccine strain
2.1.3 Hazards arising from reassortant viruses
   2.1.3.1 Direct hazards
   2.1.3.2 Indirect hazards
2.1.4 Hazards arising from the use of wild type viruses for pandemic strain vaccine production
2.2 Hazards arising from the type of production
   2.2.1 Production in eggs
   2.2.2 Production in cell cultures
2.3 Factors affecting pathogenicity for humans
   2.3.1 HA receptor specificity
   2.3.2 HA cleavability
   2.3.3 Other factors affecting pathogenicity
2.4 Hazards arising from the vaccine
2.5 Prior large scale experience with reassortants
2.6 Testing of viruses being considered for vaccine production
   2.6.1 In vivo tests to evaluate pathogenicity of H5 and H7 vaccine candidates
   2.6.2 Genetic stability of H5 and H7 vaccine candidates
   2.6.3 Evaluation of wild type non-pathogenic H5 or H7 viruses or reassortants derived from them.
   2.6.4 In vivo tests of non H5, non H7 vaccine candidates or reassortants derived from them.

3. Risk assessment
3.1 Health protection
   3.1.1 Likelihood of harm to human health
3.2 Environmental protection
   3.2.1 Nature of the work
   3.2.2 Environmental considerations
3.3 Assignment of containment level
3.4 Environmental control measures
   3.4.1 Specifications for “BSL2 Enhanced (pandemic influenza vaccine)”
      3.4.1.1 Facility
      3.4.1.2 Personal protection
      3.4.1.3 Virus monitoring
   3.4.2 Specifications for “BSL3 Enhanced (pandemic influenza vaccine)”
      3.4.2.1 Facility
      3.4.2.2 Personal protection
3.5 Biosafety management and implementation within a vaccine production facility
   3.5.1 Management structures
   3.5.2 Medical surveillance
   3.5.3 Implementation

Authors

References
Summary

International biosafety expectations for both the pilot-scale and large-scale production of human vaccines for a response to a pandemic influenza strain, and the quality control of these vaccines, are described in detail in these WHO Guidelines. Tests required to evaluate the safety of candidate influenza vaccine reference viruses prior to release to vaccine manufacturers are also specified in this document which is thus relevant to both development and production activities, and also to vaccine and biosafety regulators. A detailed risk assessment is presented that concludes that the likelihood of direct harm to human health would be high if non-reassortant H5 or H7 viruses with multiple basic amino acids at the haemagglutinin (HA) cleavage site and high in vivo pathogenicity are used for vaccine production. Such viruses could also pose a significant risk to animal health. Stringent vaccine biosafety control measures, defined as Biosafety Level (BSL)3 enhanced (pandemic influenza vaccine) are defined to manage the risk from vaccine production and quality control using such viruses in the pre-pandemic period. For all other vaccine strains, for example reassortants derived from H5 or H7 strains in which the multiple basic amino acid HA0 cleavage site has been removed, the direct risk to human health is very remote. Nevertheless, there is an indirect risk to human health due to a theoretical risk of secondary reassortment with normal human influenza viruses, resulting in a virus with avian-like coat proteins capable of replicating in humans. Although very unlikely, the secondary reassortant could become adapted to human infection and transmission which, if vaccine production was taking place in the pre-pandemic period, would have serious public health consequences. The biosafety control measures that are proposed, defined as BSL2 enhanced (pandemic influenza vaccine), take this and also potential risks to animal health into account. Facility and personal protection specifications are provided for both BSL2 enhanced and BSL3 enhanced bioafety levels and guidance is provided on biosafety management and implementation within a vaccine production facility. Tests to be performed on candidate vaccine reference strains prior to release to vaccine developers depend on the type of virus but include, at a minimum, in vivo tests on ferrets or other susceptible mammals, and, where appropriate, chickens and egg embryos, plaque assays and sequencing.

Glossary

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

_Aerosol_

A dispersion of solid or liquid particles of microscopic size in a gaseous medium.
Air balance
The necessity to keep air supply and exhaust systems in balance by means of measurements of static pressure, fan and motor performance, and air volumes.

Airlock: Areas found at entrances or exits of rooms that prevent air in one space from entering another space. These generally have two doors and a separate exhaust ventilation system. In some cases a multiple-chamber airlock consisting of two or more airlocks joined together is used for additional control.

Biosafety committee
An institutional committee of individuals versed in the subject of containment and handling of infectious materials.

Biosafety level 2 (or 3) (enhanced pandemic influenza)
A specification for the containment of pandemic influenza during vaccine manufacture and quality control testing with specialized air handling systems, waste effluent treatment, immunization of staff, specialized training, and validation and documentation of physical and operational requirements.

Biosafety manual
A comprehensive document describing the physical and operational practices of the laboratory facility with particular reference to infectious materials.

Biosafety officer
A staff member of an institution who has expertise in microbiology and infectious materials, and has the responsibility for ensuring the physical and operational practices of various biosafety levels are carried out in accordance with the standard procedures of the institution.

Biological indicators
The use of organisms to test the efficacy of sterilization processes.

Biological safety cabinet
Primary and partial containment work enclosure used for manipulation of materials that may cause infections or sensitization to workers. They are equipped with high-efficiency particulate air (HEPA) filters and may or may not be open-fronted.

Certification
Documentation that a system qualification, calibration, validation, or revalidation has been performed appropriately and the results are acceptable.

Decontamination
A process by which an object or material is freed of contaminating agents.
Floor dams
Purpose-built elevations to enclose liquid spills.

Fumigation
The process whereby gaseous chemical is applied to an enclosed space for the purpose of sterilizing the area.

Good manufacturing practices
That part of quality assurance which ensures that products are consistently produced as controlled to the quality standards appropriate to their intended use and as required by the marketing authorization.

HEPA filter
A filter capable of removing at least 99.97% of all particles with a mean aerodynamic diameter of 0.3 micrometres.

Inactivation
To render an organism incapable of replication by application of heat, or other means.

Seed lot
A culture of microorganism distributed from a single bulk container in a single operation, in such a manner as to ensure uniformity and stability and to prevent contamination.

Positive pressure laminar flow hood
An enclosure with unidirectional outflowing air, generally used for product protection.

Primary containment
A system of containment, usually a biological safety cabinet or closed container, which prevents the escape of a biological agent into the immediate working environment.

Respirator
A respiratory protective device with an integral perimeter seal, valves and specialized filtration, used to protect the wearer from toxic fumes or particulates.

Risk analysis
A formalized documented process for analysing risks.

Secondary containment
A system of containment, usually involving specialized air-handling, airlocks and secure operating procedures, which prevents the escape of a biological agent into the external environment or into other working areas.
**Sterilization**

Sterility is the absence of viable microorganisms. In general, an item is assumed to be sterile if the validation of the sterilization process applied to it indicates that only one item in one million items subjected to the process will contain a viable microorganism.

**Validation**

The documented act of proving that any procedure, process, equipment, material, activity, or system actually leads to the expected results.

**Introduction**

The earlier WHO guidance *Production of pilot lots of inactivated influenza vaccines from reassortants derived from avian influenza viruses. An interim biosafety risk assessment* (2) was prepared in response to the threat of a pandemic posed by the highly pathogenic H5N1 avian influenza viruses and the need to begin development of experimental vaccines. This threat persists and several countries are now planning large-scale production of H5N1 vaccine. The risk assessment that informed the WHO biosafety guidance for pilot-lot vaccine production (2) has therefore been reassessed in light of the intended greater scale of vaccine production and because production facilities are likely to be different from those used in developing small pilot lots, and also taking into account the experience gained from developing and testing vaccine reference viruses derived by reverse genetics from highly pathogenic avian influenza viruses.

This document follows the risk-assessment scheme used in the WHO biosafety guidance for pilot-lot vaccine production, but is extended to include considerations relating to the greater production-scale needed to supply large quantities of vaccines. The risks associated with large-scale production are likely to be different from pilot lots, e.g. the “open” aspect of some production processes and quantity of virus-containing waste. It also takes into account the considerable experience gained from highly pathogenic avian influenza viruses, and the hazards associated with such strains.

Furthermore, the range of options for vaccine development is broader than originally considered in the WHO risk assessment for pilot lot production and the present document has been expanded to encompass current vaccine development pathways.

1. **Scope of the risk assessment**

Much effort has recently gone into the development of H5N1 vaccine and manufacture and the guidance presented is strongly influenced by the experience gained with this strain and our greater knowledge of H5
strains in general. It is, nevertheless, intended that the guidance will also be applicable to future threats from other potential pandemic strains, such as H2 or highly pathogenic H7.

There is a range of possible pathogenicities in the viruses used in candidate vaccine production not only for humans but also for other mammals and avian species. On the one hand, H5 viruses that can be highly pathogenic to both humans and chickens have been used to produce reassortant viruses genetically modified to be of low pathogenicity for chickens and mammals. On the other hand, for strains inherently less pathogenic for humans, wild-type virus might be used directly for vaccine production. Thus reassortants derived by reverse genetics, empirically-derived reassortants, which may or may not be genetically modified, and native wild strains are within the scope of these guidelines.

Eggs have traditionally been used for the production of influenza vaccines, but cell culture techniques have been recently introduced and international expectations for production and quality control specifications defined (3). For the development of pandemic vaccine, either method may be used; thus both egg and cell culture production methodologies are within the scope of this document.

Most effort to date with candidate pandemic vaccine development has been targeted towards inactivated vaccines. In one country however two live attenuated virus vaccines for potential pandemic strains are under development. This may raise important issues beyond the risks to humans, namely the potential for excreted viruses or their derivatives to infect and replicate in non-human species particularly in those raised for commercial purposes. As the detection of H5 and H7 influenza strains are notifiable strains to the Office International des Epizooties (OIE), widespread dissemination of such vaccine strains could have a significant economic impact as well as ramifications for international trade. Developers and regulators will need to assess both the human and the agricultural risk of live pandemic strain vaccines under development should shedding and replication be possible. Both vaccine types (inactivated and live) are therefore covered in the scope of these guidelines.

Furthermore it is intended that the risk assessment and the guidelines on containment measures should apply to all facilities and laboratories that have a need to handle live vaccine virus. This includes not only the vaccine manufacturing facility but also to the quality control laboratories of the manufacturer and, if appropriate, to National Control Laboratories. The transport of live virus materials within and between sites should comply with international specifications (4).

Finally it should be noted that the risk assessment for vaccine manufacture will vary according to whether production is occurring in an interpandemic
period, in a pandemic alert period (as for example early in 2004 when H5N1 was threatening to circulate extensively in South East Asia) or in a pandemic period. These guidelines are intended to describe steps to minimize the risks associated with the production and testing of vaccines with emphasis on the interpandemic period, while indicating modifications that may be found appropriate during other periods.

2. Hazard identification

Hazards associated with pandemic vaccine manufacturing and laboratory testing are dependent on the type of pandemic vaccine strain (reassortant or wild type), method of production (egg-based or cell-based) and whether it is an inactivated or live attenuated virus vaccine. The type of vaccine strain, the proposed testing schedule and containment level are illustrated in Table 1.

2.1 Hazards associated with the type of pandemic vaccine virus

2.1.1 Hazards associated with the recipient virus in a reassortant strain

Pandemic vaccine reassortants have been produced on the human strain A/PR/8/34 (PR8) as recipient virus. PR8 has had over 100 passages in each of mice, ferrets and embryonated chicken eggs. The result of such a passage history is complete attenuation of the virus and its inability to replicate in humans (5).

PR8 reaches a high titre in embryonated chicken eggs and since the late 1960s, it has been used to produce “high growth reassortants” in combination with the prevailing influenza A vaccine strain. The use of such reassortants as vaccine strains has increased vaccine yield many-fold. The reassortants are produced by a mixed infection of eggs with PR8 and the nominated vaccine strain, combined with a selection system based on anti-PR8 antibody and growth at high dilution.

Live attenuated influenza vaccines are licensed in some countries. The parental strains used in such vaccines, e.g. A/Ann Arbor/6/60, are also potential recipient strains for the development of pandemic reassortant vaccines. These parental strains possess phenotypic markers of vaccine safety, such as temperature sensitivity, cold-adaptation and attenuation in ferrets or rodents and moreover have a demonstrated attenuated phenotype in humans.

2.1.2 Hazards arising from the inserted gene product in a reassortant vaccine strain

The products of the inserted genes will be, at a minimum, the haemagglutinin (HA) and neuraminidase (NA) of the pandemic strain virus. For reassortants derived from highly pathogenic H5 or H7 strains by reverse genetics, the
HA will have been modified so that the multiple basic amino acids at the HA cleavage site, which are associated with high pathogenicity, will be reduced to a single basic amino acid. Any protein derived from the wild-type strain on its own will be neither inherently infectious nor harmful.

2.1.3 Hazards arising from reassortant viruses

2.1.3.1 Direct hazards

Without treatment, reassortant viruses may be expected to survive for at least a short time (hours) on surfaces or in a laboratory environment and thus provide a potential means of infection for laboratory workers. Although the surface antigens of reassortants, particularly the HA, can contribute to pathogenicity (5, 6) published information indicates that a reassortant between PR8 and a wild-type human influenza virus is likely to be avirulent in humans (5, 7–9). Although such information is difficult to interpret because the genetic composition of the reassortants was not clear, it is known that the degree of attenuation increases as reassortants include more PR8 genes (10, M Tashiro, unpublished data). The reassortants created by reverse genetics as H5N1 pandemic reference strains contain six out of eight viral genes from PR8 and the NA and modified HA genes of the H5N1 virus. Furthermore, the H5 HA retains a preference for α2,3 linked residues (see below), so the ability of the H5N1 reassortants to bind to and replicate in human cells should be minimal. It is therefore envisaged that an H5N1 reassortant derived by reverse genetics according to WHO guidance (11) would be attenuated for humans compared to the H5 wild type. Furthermore, it is clear that such reassortants are expected to be of low pathogenicity in chickens and other animals compared to the highly pathogenic parental wild strains, and this expectation has been borne out by experience to date. Nevertheless, as the factors affecting pathogenicity are not fully understood (see below), genetic manipulation to remove the polybasic sites could theoretically have unpredicted effects on both transmissibility and pathogenicity.

For reassortants derived by traditional co-cultivation methods, the gene constellation is less predictable. There is a theoretical possibility of developing reassortants with more than two wild-type parental genes or even of selection of a mutant (non-reassortant) wild-type virus with improved growth characteristics. If vaccine production takes place in the interpandemic phase there would be a need to determine the gene constellation of reassortants derived by traditional co-cultivation methods in order to conduct a full risk assessment.

Reassortants with a 6:2 gene constellation based on live attenuated recipient strains such as A/Ann Arbor/6/60, or other strains used as live attenuated vaccines, may also be used for the production of pandemic influenza vaccine. The attenuated A/Ann Arbor/6/60 strain has been used
as a backbone in 6:2 reassortant live attenuated vaccines in clinical studies for more than 30 years using approximately 30 different vaccine strains, and the data demonstrate that the Ann Arbor/6/60 virus produces reassortant vaccine strains that are attenuated for humans (12). Live vaccines derived from the Ann Arbor strain have been licensed in one country. An adequate level of attenuation should be expected for modified H5 reassortant strains. For each candidate pandemic strain, this should be verified by testing as described below (section 3.6.1).

Reassortants may be also be derived from non-H5 or non-H7 viruses (e.g. H9N2, H2N2) and may use either PR8 or an attenuated vaccine strain. The hazards associated with such reassortants depend on HA receptor specificity. If a reassortant has a preference for avian cell receptors (α2,3 linked sialic acid e.g. avian H2N2 viruses), the hazards are considered to be no different from those associated with the above-mentioned 6:2 reassortants derived from attenuated H5 or H7 viruses (see section 3.3). However, if a reassortant has a preference for mammalian cell receptors (α2,6 linkages, e.g. human H2N2 pandemic virus from 1957), or possesses both avian and mammalian receptor specificities (e.g. H9N2), there is a greater risk of human infection (see Table 1).

2.1.3.2 Indirect hazards

Although it is considered that, for example, an H5N1/PR8 reassortant will be either attenuated or possibly non-infectious to humans, an indirect hazard may exist through secondary reassortment with a human or animal influenza virus as influenza viruses are known to exchange genes by the process of reassortment. For secondary reassortants to be generated, several events need to occur; firstly infection of the production staff with the reassortant strain; secondly, for the infected worker to have a mixed infection with a wild type influenza virus, and thirdly for a reassortment event to take place. In practice, manufacturers have 30 years of experience with large scale production of vaccines based on PR8 reassortants and there have been no reported cases of human illness. However, it should be noted that this does not rule out the possibility of infections having occurred. Additionally, at the point when seasonal influenza vaccines become available, production staff can be vaccinated to reduce the chances of an infection with a circulating wild-type virus.

In practice, the lack of success in producing H5N1 reassortant vaccine strains in 1997 (UK: avian and swine viruses; Australia and USA: avian and PR8 viruses) suggests that the probability of producing H5 reassortants between mammalian and avian viruses in human cells is slight. It should also be considered that poultry and pig farmers are continually exposed to animal influenza viruses and there have been few documented cases of human infection in this population with a reassortant between an avian or
porcine and a human influenza virus. Based on these considerations the probability that a PR8 reassortant strain will replicate and combine with another influenza virus(es) in human cells is considered to be minimal. The risk of such secondary reassortments for animal species will be considered in the environmental risk assessment section (see section 3.2).

2.1.4 **Hazards arising from the use of wild type viruses for pandemic strain vaccine production**

Wild-type strains may be considered for production purposes and different potential vaccine candidates could be:

— an avian strain with no record of human infection (surrogate virus);
— an avian strain with documented human infection (potential pandemic virus);
— an actual human pandemic virus, or a past H2N2 pandemic virus.

The hazards from wild type vaccine strains will differ according to the category of wild-type virus used but in all cases are compounded during vaccine manufacturing and associated vaccine product testing, due to the high volumes and high titres encountered. With the exception of surrogate viruses, the use of wild type pandemic-like influenza viruses to develop pandemic vaccine strains presents considerable biosafety risks to personnel in vaccine manufacturing facilities and testing laboratories, and also to the general community if manufacture is taking place for clinical studies or stockpiling of vaccines during the interpandemic period.

2.2 **Hazards arising from the production process**

Vaccine manufacture follows *Good manufacturing practices for biologicals* (13). Good manufacturing practices (GMP) require protection of the product from the operator and the environment and thus amelioration of certain hazards associated with production will require the establishment of a suitable balance between GMP and biosafety requirements.

2.2.1 **Production in eggs**

Influenza vaccine has been produced in embryonated hens’ eggs on a large scale since the early 1950s. Much experience has been gained and some facilities are capable of handling large numbers of eggs on a daily basis with the aid of mechanized egg handling, inoculation and harvesting machines.

Hazards occur only during the production stages and quality control laboratory activities prior to virus inactivation. The most hazardous production stage is egg harvesting when the eggs have to be opened to harvest the allantoic fluid. The volume and titre of virus is higher at this
stage than at any other. The open nature of the operations leads to a greater exposure to aerosols and spills. In contrast during egg inoculation, the virus used is dilute and of a relatively small volume. The allantoic fluid that is harvested from the eggs is invariably manipulated thereafter in closed vessels and hazards arising from live virus during downstream processing and during the virus inactivation process, if used, are therefore less than during virus harvest. Collection and disposal of egg waste is potentially a major environmental hazard. Safe disposal of the waste from egg-grown vaccines, both within the plant and outside, is therefore critical.

2.2.2 Production in cell cultures

For pandemic influenza vaccines produced on cell cultures, the biosafety risks associated with manufacturing will depend primarily on the nature of the cell culture system employed. Closed systems, such as bioreactors, normally present little to no opportunity for exposure to live virus during normal operation, but additional safety measures must be taken during procedures where samples are introduced into or removed from the bioreactor, and during procedures to deal with accidental spills. Roller bottles and cell culture flasks used for virus production may allow exposure to live virus through aerosols, spills, and other operations during virus production and, thereafter, additional risks are associated with the inactivation and disposal of the large quantities of contaminated solid waste generated by this method.

The possibility exists that genetic mutations may be selected in pandemic vaccine viruses during passage in mammalian cells that render them more adapted to humans. Sequence analysis of the region of the HA gene encoding the receptor binding site may be useful. However, it should be noted that little is known about the relation between cell substrate and virus reversion or adaption. Beare et al. (5) tried to de-attenuate PR8 by multiple passage in organ cultures of human tissue, but failed, whereas studies with MDCK cells (14) demonstrated that human viruses that retained their α2,6 receptor specificity (human-like) were likely to mutate to an α2,3 specificity (avian-like) as this provided a replicative advantage on MDCK cells, rather than the reverse. Overall, hazards arising from the inherent properties of a reassortant or wild type virus are likely to be far greater than the probability of adaptation of the virus to a more human-like phenotype.

2.3 Factors affecting pathogenicity for humans

2.3.1 HA receptor specificity

The influenza HA is responsible for attachment of virus to the target cell and has specificity for sialic acid receptors on cell surface molecules. The HAs present on human influenza A viruses preferentially bind to receptors
containing α2,6-linked sialic acid residues, whereas avian influenza viruses preferentially bind to α2,3-linked sialic acid (15). Human tracheal cells have mainly α2,6 linked residues (16), so the acquisition of an avian HA by PR8 virus is expected to minimize potential binding to human respiratory epithelial cells. Although the α2,3 receptor specificity of avian viruses will reduce the efficacy of such binding, it may not completely prevent infection in humans. Moreover, the presence of avian-like receptors has been demonstrated in human respiratory tract epithelium (17). Beare and Webster (18) found that over 100 fold higher quantities of avian viruses (between 106.8 and 109.2 egg infectious doses) were needed for replication in humans and, because replication was poor, that it was not possible to induce person-to-person transmission.

There have been many reports of human infections with avian H5N1 viruses since 1997 in south-east Asia. It is possible that exposure to high-titre H5N1 virus in contaminated chicken or duck carcasses or animal products may have overcome the avian specificity of HA receptor binding. Virus replication in such human cases was much better than in the earlier experimental studies of avian influenza viruses in humans (18); however, the extensive replication of H5N1 viruses in these people is inexplicable on the basis of current knowledge of receptor specificity because the viruses isolated from them retained the α2,3 avian specificity.

2.3.2 HA cleavability

The HA of influenza virus must be cleaved into HA1 and HA2 by host cell proteases as a prerequisite for infectivity, and this cleavage has been correlated with virulence. The pathogenicity of H5 and H7 influenza A viruses in chickens is largely determined by the nature of the amino acids at the HA cleavage site. H5 and H7 viruses with multiple basic amino acid sequences are highly pathogenic and their HA can be effectively cleaved by the ubiquitous furin-like proteases, which are expressed in most organs of birds and humans. In contrast, the HA of H5 and H7 viruses of low pathogenicity for birds and certain laboratory animals contain a single basic residue at the cleavage site, a feature common to all other subtypes of influenza HA, and which can only be cleaved by trypsin-like proteases, which are restricted to certain cell types, e.g. epithelial cells lining the respiratory tract of humans and the gut of birds. Thus, HA cleavability influences tissue specificity and is a major determinant of pathogenicity for H5 and H7 viruses in chickens and certain laboratory animals. Multiple basic amino acids at the cleavage site have not been observed for any other HA subtype.

Direct evidence has been obtained that both HA cleavage and HA receptor specificity have an effect on tissue tropism of an avian H7N1 virus, A/
Fowl Plague/Rostock/34 in chicken embryos (19). Similarly, the available evidence from the H5N1 infections in 1997 demonstrates that the high degree of pathogenicity in chickens, mice and ferrets is directly influenced by the presence of the multiple basic amino acids. Webby et al. (20) demonstrated that removal of the basic amino acids changed H5N1 infections from a fatal systemic infection to a localized non-pathogenic infection in chickens (i.e. low pathogenicity for chickens), mice and ferrets. Hatta et al. (21) and Lipatov et al. (22) have also shown by reverse genetics that high cleavability of H5N1 HA due to the presence of multiple basic amino acids was an essential requirement for a lethal mouse infection. It is not ethical to examine the pathogenicity of influenza virus infection in humans, but an examination of H5N1 viruses by Gao et al. (23) provided evidence that pathogenicity in mice can resemble that in humans. The occurrence of multiple organ failure after human H5N1 infections is suggestive of an unusual tissue tropism. Although evidence for viral replication outside the lung has been described for at least one human case (24), such evidence remains difficult to document (25).

The available evidence suggests that virulence of the 1997 and later H5N1 viruses for humans is related to the presence of the HA multiple basic amino acids. It is therefore considered imperative to remove them, if present in the HA of any H5N1 virus being developed as a vaccine strain, to reduce the potential for harm to humans. This procedure will also increase the safety of the reassortants for avian species (see below under environmental risk assessment) as cleavage site modifications have resulted in a reduction of their pathogenicity in avian embryos (26). It should be noted that during production of reassortants by reverse genetics, base substitutions are introduced to stabilize the removal of multiple basic amino acids during passage of reassortants.

2.3.3 Other factors affecting pathogenicity

Although it is clear from experience in south-east Asia from 1997 to the present that H5N1 influenza viruses that display α2,3 sialic acid specificity could replicate in humans, it must be noted that influenza virus pathogenicity does not depend solely on HA, but is a polygenic trait. The 1997 H5N1 virus had unusual PB2 and NS1 genes that influenced pathogenicity whereas the 2004 H5N1 viruses possess complex combinations of changes in different gene segments that affect pathogenicity in ferrets (27). Changes in the PB2 gene of the 1997 H5N1 viruses were sufficient to attenuate them for mice (21) and changes in the NS1 protein rendered these viruses resistant to the effects of interferons and other cytokines produced as part of the innate immune response (28). The changes to NS1 conferred a highly virulent phenotype which allowed replication to proceed unchecked in vivo. In this case even a virus with a poor affinity for its receptor was able to replicate
(although not to transmit). In contrast, viruses with a gene constellation producing PR8 internal proteins were clearly sensitive to the innate immune mechanisms which prevent the establishment of infection by an avian virus in humans. This may well explain why in the outbreaks of H5 avian influenza before 1997, no evidence of transmission from birds to humans was noted. Further, prior to the 2003 outbreak in the Netherlands, only two cases of transmission of H7 viruses from birds to humans were documented (29, 30). Also during the many years of laboratory handling of high-titre avian viruses (of which one H7 strain (A/FPV/Dobson) is known to contain a gene which adapts it for replication in mammalian cells (31)), there has only been one report in the literature of a worker being affected by these viruses. This was a laboratory worker in Australia who developed conjunctivitis after accidentally being exposed to a H7N7 virus directly in the eye (32). The PR8/H5N1 6:2 reassortants and the A/Ann Arbor/6/60 live attenuated 6:2 reassortants created by reverse genetics for the production of H5N1 vaccine do not contain the gene constellation considered necessary for pathogenicity in chickens, mice and ferrets and in contrast have internal genes that confer sensitivity to the innate immune response.

### 2.4 Hazards arising from the vaccine

Inactivated pandemic influenza vaccines present no biosafety risks provided that the results of the inactivation steps show complete virus inactivation, as the viral vaccine is rendered incapable of replication.

In an interpandemic or pandemic alert period, pilot-scale live attenuated pandemic influenza vaccines may be developed for clinical evaluation. As there is some uncertainty concerning the biosafety risks associated with shedding or other unintentional release into the environment following vaccination, subjects participating in clinical trials in the interpandemic or pandemic alert phase should be kept under appropriate clinical isolation conditions. If this is not done, indirect hazards for humans could arise as considered in section 3.1. Furthermore, for pandemic human influenza vaccine strains that express H5 or H7 avian influenza genes, there will be potential consequences for agricultural systems (section 3.2.2). If viruses of the H5 or H7 subtype become transmissible in livestock, this would be notifiable to OIE and could result in sanctions with serious economic and trade implications to prevent the spread of disease.

If a human pandemic has already started, the hazards from live attenuated vaccines elaborated above will not be relevant.

### 2.5 Previous large-scale experience with reassortants

Reassortants derived from PR8 have been used routinely for the production of inactivated influenza vaccines for the past 30 years. This work involves
the production of many thousands of litres of infectious egg allantoic fluids, which create substantial aerosols of reassortant virus within manufacturing plants. Most of the reassortants were made from wild type human strains that had not yet been in widespread circulation. Thus, although the manufacturing staff would have some susceptibility to infection with the wild type virus, there have been no anecdotal or documented cases of work-related human illness resulting from occupational exposure to the reassortants.

Similarly, reassortants derived from the A/Ann Arbor/6/60 strain have been used for the production of live attenuated vaccine for at least 3 years and no anecdotal or documented cases of work-related human illness have been reported. While to date no conclusive study has been conducted to detect silent infections for either the PR8 or live attenuated strains, and thus infectivity in humans cannot be fully assessed, the attenuation status of these vaccine strains continues to be supported by their excellent safety record to date.

However, unlike the situation with the human influenza strains selected for the annual vaccine formulation, staff manufacturing an H5N1 vaccine would have no previous immunological experience of the avian virus, and would therefore be expected to be susceptible, although the risk of work-related human illness and of transmission outside the facility is expected to be slight and lower than for non-reassortant strains.

### 2.6 Testing of reference viruses being considered for vaccine production

Vaccine reference viruses will be developed by a WHO laboratory or by a laboratory approved by a national regulatory authority (hereafter, for ease of reference, referred to as a WHO laboratory). The following tests and specifications have been developed based on experience gained in the evaluation of 6:2 reassortant H5N1 viruses produced on the PR8 and A/Ann Arbor/6/60 backbones. The principles outlined should be applicable during the interpandemic period to other reassortant strains, but exceptions may be made if appropriately justified. Tests on wild-type viruses being considered for vaccine production will need to be selected on a case-by-case basis. The tests described below are usually conducted by the WHO laboratory developing the reference strain.

In a pandemic alert period or a pandemic period, the requirement for the conduct or the completion of some or all of these tests prior to the distribution of a candidate reference strain may be relaxed based on the risk assessment. For example, in a pandemic alert period, a candidate reference strain which on the basis of molecular analyses, is expected to have a low risk of human infection and transmission could be distributed to vaccine manufacturers to enable them to begin preparation of their seed stocks prior
to the completion of time-consuming tests such as the chicken and the ferret pathogenicity tests. If a pandemic has already begun, and the pandemic virus has become adapted to human infection, there may be no need to perform all the pathogenicity tests indicated below. A risk assessment should be performed for each candidate reference strain and the outcome will depend on the nature of the strain and the pandemic period declared by WHO.

2.6.1 In vivo tests to evaluate pathogenicity of H5 and H7 viruses

For optimal interpretation of tests, the pathogenic properties of the candidate reference virus, should be compared with those of the parental backbone strain and the wild-type strain.

These tests should be performed under appropriate high laboratory containment conditions (see section 4.3). Tests to be performed on the candidate vaccine reference strain (see Table 1) by the WHO reference laboratory that develops the reassortant strain include:

- **The ability to plaque in the presence or absence of added trypsin.** Viruses with high pathogenicity can replicate in mammalian cell culture in the absence of added trypsin, whereas those with low pathogenicity generally do not.
- **The ability to cause chicken embryo death.** Highly pathogenic viruses cause rapid chicken embryo death upon inoculation into eggs whereas removal of the multiple basic amino acids from a highly pathogenic strain results in embryo survival (26).
- **Pathogenicity in chickens.** The chicken intravenous pathogenicity (IVP) test is an important statutory test required by veterinary authorities, and a reassortant virus must have an index of 1.2 or less before it can be removed from high-level containment (33). Development of specifications to indicate that the test articles have been correctly administered in the IVP test would be beneficial.
- **Attenuation in ferrets.** The viruses should be shown to be attenuated in ferrets or in other suitable animal models, provided they have virus sensitivity equivalent to that of ferrets and a similar ability to discriminate between highly pathogenic and non-pathogenic influenza viruses. These tests compare the candidate reference virus with the wild type virus. Detailed test procedures are described in Appendix 1. In the case of H5N1 reassortants, the criteria used to evaluate this test are that virus replication and clinical symptoms should be comparable to those induced by the attenuated PR8 parent virus and should be milder than the wild-type human H5N1 virus infection.

Ferrets were chosen because they have been used extensively as a good indicator of influenza virus virulence for humans (reviewed by Smith and Sweet, 34). Typically, human influenza viruses cause lethargy, nasal discharge and occasionally fever in ferrets, and virus replication is usually
limited to the respiratory system. PR8 virus has been assessed in ferrets and found to cause few or no clinical signs, and virus replication is limited to the upper respiratory tract. However, the 1997 and 2004 wild-type human H5N1 viruses replicated in ferrets throughout the body, caused fever, weight loss and occasionally death (27, 35). Thus, in the absence of human data, the ferret is the best model to predict whether a virus will be pathogenic or attenuated in humans.

It would be useful to be able to measure transmissibility as well as pathogenicity of virus strains, but currently a well-characterized methodology to do so is lacking. Intranasal administration of virus to chickens may be one such method, and has been shown to be possible, but to date the test is not standardized. Uninoculated birds in close contact with infected birds in the intravenous pathogenicity test may provide some information on transmissibility. Transmission studies in ferrets after oral and ocular inoculation are also potentially useful, but need to be standardized.

Tests for safety in mice may provide useful information if the parent strain is virulent in mice. Detailed test procedures are described in Appendix 1.

A reassortant virus should be used for vaccine manufacture only after appropriate results have been obtained in the above tests. For H5 and H7 strains, the nucleotide sequence corresponding to the HA cleavage site should be determined by the WHO laboratory to demonstrate the absence of multiple basic amino acids in the vaccine candidate. After WHO has declared a pandemic manufacturers may receive candidate reference strains that have not been assessed fully for pathogenicity. In this case they should handle the viruses appropriately depending on the nature of the virus and the pandemic situation.

2.6.2 Genetic stability of H5 and H7 viruses

Genetic stability is an important issue as it is known that in poultry, wild-type low-pathogenicity H5 and H7 avian viruses can become highly pathogenic by mutation (insertion of basic amino acids at the HA cleavage site) and this is the origin of the highly pathogenic H5 and H7 strains. Although the derivation of low-pathogenicity candidate reference viruses by reverse genetics involves the introduction of silent mutations in the region of the HA cleavage site that should minimize the re-insertion of multiple basic amino acids, during vaccine production, such viruses may be passaged several times and it is therefore important to evaluate their genetic stability at the cleavage site. Several attenuated reassortants have now been produced between PR8 virus and highly pathogenic H5N1, H5N3 and H7N1 viruses by reverse genetics (20, 26, 36, 37, FLUPAN (http://www.nibsc.ac.uk/flupan/)) and following extended passage in eggs (up to 10), they have each retained their attenuated phenotype.
Nevertheless, manufacturers should assess any H5 and H7 seed viruses and vaccine virus harvests by sequence analysis of the HA cleavage site. The need for studies of genetic stability for seed viruses prepared from candidate reference strains derived by other methods should be assessed on a case-by-case basis. At least one in vivo test (section 3.6.1) should be applied, for example the egg embryo lethality test.

2.6.3 **Evaluation of wild-type non-pathogenic H5 or H7 viruses or reassortants derived from them**

In view of the propensity for non-pathogenic H5 and H7 viruses to acquire mutations leading to increased pathogenicity, it is advisable to conduct the full spectrum of pathogenicity tests (in ferrets, chickens and chicken embryos), as indicated in section 3.6.1.

2.6.4 **In vivo evaluation of non-H5, H7 viruses or reassortants derived from them**

Ferret tests are required for non-H5, non-H7 candidate vaccine strains prior to manufacture. The tests should be conducted under biocontainment levels equivalent to that required for the production of the reference strain. The other tests (specified in sections 3.6.1 and 3.6.2) are not required because they are specific for reassortants derived from highly pathogenic H5 and H7 viruses.

3. **Risk assessment**

3.1 **Health protection**

3.1.1 **Likelihood of harm to human health**

By virtue of PR8 attenuation, avian receptor specificity, loss of multiple basic amino acids at the HA cleavage site and the absence of other H5N1 genes associated with pathogenicity in humans (i.e. NS1 or PB2 genes), it is envisaged that an PR8 x H5N1 6:2 reassortant, although possibly infectious to humans and ferrets, will have only a low probability of causing harm to human health. On the basis of these arguments, reassortants derived from H5 or H7 strains in which the multiple basic amino acid HA0 cleavage site has been removed, using either PR8 or strains attenuated for humans e.g. the A/Ann Arbor/6/60 as the recipient virus, would be likely to be similarly attenuated. Reassortants derived from all other subtypes or from low pathogenicity H5 and H7 subtypes, in which the multiple basic amino acids were not present, should also be attenuated by virtue of the receptor specificity of the avian HA and the attenuating effect of the 6 PR8 genome segments (absence of any other avian genes). The same arguments are also valid for reassortants prepared from live attenuated virus strains such as A/Ann Arbor/6/60.
If staff at a vaccine production plant are exposed to aerosols containing high-titre reassortant virus, sub-clinical infections could result. If this happened, it is very unlikely that a reassortant virus would transmit to human contacts as it is likely that replication will be attenuated and virus shedding, if it occurs, it would be well below the titres considered to be needed for human infection.

However, although there is no precedent, as described above there is a theoretical possibility of secondary reassortment with normal human influenza viruses and that such reassortant viruses may be replication-competent in humans, while having avian-virus like coat proteins. Although it is very unlikely that the secondary reassortant could become adapted to human infection and transmission, were this to happen the public health consequences would be serious. The likelihood of such occurrences can be reduced through biosafety measures designed to limit exposure of personnel to high-titre materials during vaccine production and testing.

If non-reassortant wild-type viruses with multiple basic amino acids at the HA cleavage site and high in vivo pathogenicity are used for vaccine production they would potentially be highly pathogenic and transmissible in humans. Stringent vaccine biosafety control measures are required to manage the risk from vaccine production using such viruses. Non-reassortant wild-type viruses, without multiple basic amino acids at the HA cleavage site with low in vivo pathogenicity and avian receptor specificity are likely to be less pathogenic and less transmissible in humans (18) than the wild type viruses described above. However the risks of secondary reassortment with normal human viruses remain and the risk that such reassortant viruses may be able to replicate in humans. Appropriate vaccine biosafety control measures are required to manage the risk from vaccine production using such viruses. Non-reassortant wild-type viruses, without multiple basic amino acids at the HA cleavage site, with low in vivo pathogenicity and mammalian receptor specificity (e.g. human H2N2 and H9N2) are also likely to be less pathogenic than the wild-type viruses described above, but their ability to transmit to humans is unknown. Consequently, because of the risks of secondary reassortments, appropriate biosafety control measures should be considered.

3.2 Environmental protection

3.2.1 Nature of the work

Egg-based vaccine production represents a relatively open system with several operations likely to generate virus aerosols: namely, seed virus preparation, egg inoculation, harvest of infected egg fluids, use of laminar outward air flows, segregation of contaminated eggs, cleaning (that may include high powered spraying) and decontamination of contaminated egg trays, and disposal of waste products.
Prior to the virus inactivation step, cell culture production requires handling large volumes of high-titre preparations of live influenza virus. As mentioned above, even in closed systems such as bioreactors leaks can occur, and spillage or other operator contact with high-titre viral solutions during the introduction of materials into the bioreactor, taking of samples, or clean-up procedures is possible. If roller bottles or cell culture flasks are used in place of bioreactors there will be a higher risk of generating aerosols and spills due to the increased manipulations required, and the volume of materials to be properly decontaminated for disposal will be proportionally greater.

3.2.2 Environmental considerations

Influenza A viruses are endemic throughout the world in some farm animals (pigs and horses) and some populations of wild birds, specifically birds of the families Anseriformes (ducks, geese and swans) and Charadriiformes (shorebirds) (38). Of the influenza A viruses, a number can cause disease in domestic poultry, such as H5, H7 and H9. H5 and H7 are thought to be highly pathogenic in poultry, whereas H9 is typically less so. In addition, sporadic infections by influenza A viruses have been reported in farmed mink, wild whales and seals, dogs and captive populations of big cats (tigers and leopards) (38, 39). In dogs, the influenza A infections were caused by H3N8 viruses closely related to endemic equine viruses, and in the big cats, the infections followed consumption of dead chickens infected with H5N1 viruses.

In the case of an H5N1 reassortant, the virus will have avian receptor specificity, and thus birds would theoretically be the species most susceptible. The contribution of the six PR8 internal genes to replication and virulence in birds is unknown.

However, Hatta et al. (40) have recently demonstrated, by the use of reverse genetics, that acquisition of only one PR8 gene by an avian influenza virus can abolish virus replication in ducks. Experimental evidence has demonstrated that PR8 virus is attenuated not only in humans (see above), but also chickens (37). Furthermore, a reasortant between PR8 (internal protein genes) and the 1997 Hong Kong H5N1 virus (NA and HA with a single basic amino acid) replicated poorly in chickens and was not lethal. Similar studies have been performed with the 2003 Hong Kong H5N1 virus at the WHO Collaborating Centre, Memphis, USA (R Webster, unpublished data), where the 6:2 PR8 reasortant did not replicate or cause signs of disease in chickens. The removal of the multiple basic amino acids from the H5 x PR8 reasortants in both studies probably played a major role in reducing the risk for chickens.

Although replication occurs in chicken embryos, for reasons that are unknown, the risk of environmental transmission via such replication in nature is remote.
Pigs are uniquely susceptible to infection by all strains of influenza A virus because they have both alpha 2,3 and alpha 2,6 receptors in abundance. Although pigs are not susceptible to infection with PR8, a reassortant containing a single gene (HA) from an A/New/Jersey/76 (H1N1) isolate, infected pigs and the animals excreted virus (6). It is thus conceivable that pigs are susceptible to infection by an H5N1 reassortant, as viruses with avian receptor specificity are known to replicate in this species. It is also possible that these species would be susceptible to secondary reassortments between the H5N1 reassortant and a pig virus. There is in fact evidence that triple reassortants between avian, pig and human influenza viruses have circulated in pigs (41).

### 3.3 Assignment of containment level

The production of influenza vaccine reassortant reference viruses, by WHO Collaborating Centres, from highly pathogenic H5 or H7 wild type viruses should take place at a high level of biocontainment (BSL-3 enhanced or BSL-4, as advised by WHO and national authorities) (11). The collaborating centres provide characterized reassortant reference viruses to vaccine manufacturers who may develop vaccine seeds and vaccines from these materials.

In consideration of the hazards associated with egg and cell culture H5 and H7 vaccine production and quality control with reassortant viruses of demonstrated low pathogenicity in chickens and/or in ferrets (and mice if applicable), as specified in sections 3.6.1 and 3.6.2, the assigned containment level is BSL-2 enhanced (pandemic influenza vaccine), as defined below (see Table 1). This applies to both pilot-scale and large-scale production during the interpandemic phase and pandemic alert period (42) when the site of vaccine production is geographically remote from the site of the emerging pandemic. Any subsequent relaxation of the levels of containment during the developing pandemic, should be decided on a case-by-case basis after careful evaluation of the risks.

In consideration of hazards associated with egg and cell culture vaccine production and quality control with wild-type viruses (non-H5 and non-H7) of demonstrated low pathogenicity in ferrets, as specified in section 3.6.3, the assigned containment level is BSL-2 enhanced (pandemic influenza vaccine), as defined below. This applies to both pilot-scale and large-scale production during the interpandemic phase and pandemic alert period (42) when the site of vaccine production is geographically remote from the site of the emerging pandemic. Any subsequent relaxation of the levels of containment during the developing pandemic, should be decided on a case-by-case basis after careful evaluation of the risks.

In consideration of hazards associated with cell culture vaccine production and quality control with highly pathogenic H5 or H7 wild-type viruses, the
assigned containment level is BSL-3 enhanced (pandemic influenza vaccine), as defined below. This applies to both pilot-scale and large-scale production during the interpandemic phase and pandemic alert period (42) when the site of vaccine production is geographically remote from the site of the emerging pandemic. Any subsequent relaxation of the levels of containment during the developing pandemic, should be decided on a case-by-case basis after careful evaluation of the risks. In addition, the parts of the facility where such work is done (both production and quality control) should meet the OIE requirements for containment, which include not only biosafety, but also requirements for biosecurity. (33). In view of the open nature of large scale egg-based vaccine production, it is not possible to operate at BSL-3 enhanced (pandemic influenza vaccine). Therefore egg-based vaccine production from high pathogenicity H5 or H7 wild-type strains is not recommended.

For vaccine production and quality control using other types of vaccine virus (e.g. reassortants derived from non-H5 or H7 viruses; wild-type low-pathogenic H5 or H7 viruses), the assigned containment level is BSL2 enhanced (pandemic influenza vaccine), as defined below. This applies to both pilot-scale and large-scale production during the interpandemic phase and pandemic alert period (42) when the site of vaccine production is geographically remote from the site of the emerging pandemic. Any subsequent relaxation of the levels of containment during the developing pandemic, should be decided on a case-by-case basis after careful evaluation of the risks.

It should be noted that implementation of the containment conditions described in this section within a production and quality control testing facility must take into account the large quantities and high titres of live virus that are produced, the industrial scale of facilities, as well as the rules and regulations governing the manufacture and testing of medicinal products known as good manufacturing practices (GMP) (13). The facility requirements for a specific biosafety level within a manufacturing plant will differ from the facility requirements within a laboratory handling smaller quantities of infectious material such as a laboratory producing reassortant reference viruses or in a pilot-scale facility. It should also be noted that these biosafety requirements apply to the production and quality control operations involving live viruses; virus lots shown to be inactivated by a validated process need not be handled under these conditions.

3.4 Environmental control measures

Each vaccine manufacturer must review their own control measures in light of the intended work, the nature of laboratory and production facilities and the need to maintain GMP. Influenza specific enhanced containment measures (defined in 4.4.1 and 4.4.2) should be in place for open manipulations with live
virus, especially virus harvesting in egg production facilities. Quality control facilities need to meet production containment requirements, and in some regions, a second approval will be needed to meet other requirements such as those regulating products containing materials derived from a genetically modified organism (GMO).

Local safety regulations provide guidance on the disposal of potentially infectious waste. Contaminated waste from current production facilities may reach high virus titres. Decontamination methods should be validated. If possible, decontamination of waste should take place on site. Where this is not possible, there should be procedures in place to ensure that material is safely contained and transported prior to decontamination off site. Guidance on regulations for the transport of infectious substances is available from WHO (4). In all cases the procedures should be validated to ensure that they function at the scale of manufacturing.

In view of the possible exposure to high titre pandemic strain virus and the need to reduce the chance of simultaneous infection with human influenza viruses, staff should be prophylactically vaccinated with seasonal influenza vaccines. It is anticipated that before large scale vaccine production is attempted, pilot lots of pandemic strain vaccine will have already been produced. Experimental vaccines inducing protective antibody levels are recommended for use by staff before large scale vaccine production commences if possible. Antiviral treatment must be available in case the situation warrants it.

Each manufacturer should also assess the risk of contamination of birds or pigs based on the likelihood of their being in the vicinity of the manufacturing plant, and the manufacturing controls in use. Staff or other personnel entering the area potentially exposed to live virus should avoid visiting pig, horse or bird facilities (e.g. farms, equestrian events, bird sanctuaries) for at least 14 days following occupational exposure. This period should be extended to 14 days after the symptoms resolve if conjunctivitis or respiratory signs indicating the potential development of influenza infection or disease develop during this 14 day period.

It is also known that mice can be experimentally infected with some influenza viruses and the PR8 strain is known to be lethal for mice. It is not known whether a reassortant based on PR8 will be able to replicate in mice, but steps should be taken to prevent exposure of wild mice and the escape of laboratory mice, and rodent control measures should be in place.

3.4.1 Specifications for “BSL2 enhanced (pandemic influenza vaccine)”

Specifications for BSL2 enhanced (pandemic influenza vaccine) facilities include the following in addition to the principles for BLS2 facilities as specified in the WHO Laboratory biosafety manual (1).
3.4.1.1 *Facility*

The facility should be designed and operated according to the stage of the manufacturing process to meet the demands of protection of the recipient of the vaccine, the staff producing and testing the vaccine and of the environment. It is noted that different solutions may be needed depending on the risks inherent in the operation(s) conducted in an area. Specialized engineering solutions will be required that may include:

— use of relative negative pressure biosafety cabinets when possible;
— use of high-efficiency particulate air (HEPA) filtration of air prior to exhaust into public areas or the environment; and
— use of positive pressure with negative pressure in-line sinks prior to exhausting to the non-viral zone.

In addition the following decontamination procedures should take place:

— decontamination of all waste from BSL-2 enhanced (pandemic influenza vaccine) areas; and
— decontamination of manufacturing and quality control areas at the end of a production campaign through cleaning and validated decontamination for example gaseous fumigation.

3.4.1.2 *Personal protection*

- Full-body protective laboratory clothing (for example Tyvek® disposable overalls) is to be worn in the controlled BSL-2 enhanced (pandemic influenza vaccine) area.
- If activities cannot be contained by primary containment and open activities are being conducted, the use of respiratory protective equipment, such as N95, FFP3 (43) or equivalent respirators is strongly recommended. Minimal specifications for the filtering/absorbing capacity of such equipment should be met, and masks, if used, must be fitted properly and the correctness of fit tested.
- Personnel should be instructed, in a written document to which they sign their agreement, not to have any contact with birds or pigs, in particular farm animals for 14 days after departure from the facility where vaccine has been produced. Currently the risks involved in contact with household dogs and cats are not considered to be significant, but the available scientific evidence is sparse.
- Staff should be prophylactically vaccinated with seasonal inactivated influenza vaccines.
- It is anticipated that before large scale vaccine production is attempted, pilot lots of pandemic strain vaccine will have already been produced. Experimental vaccines inducing protective antibody levels are recommended for use by staff before large scale vaccine production commences if possible.
- Antiviral treatment must be available in case the situation warrants it.
3.4.1.3 **Monitoring of decontamination**

- Cleaning and decontamination methods need to be validated periodically as part of a master validation plan to demonstrate that the protocols, reagents and equipment used are effective in the inactivation of pandemic influenza virus on facility and equipment surfaces, garments of personnel and waste materials, and within cell growth and storage containers. Once decontamination procedures for influenza virus have been fully described and validated, there is no need to repeat them for each new strain. Validation studies using influenza viruses may be supplemented by studies with biological (for example bacterial) markers selected to be more difficult to inactivate than influenza.

3.4.2 **Specifications for “BSL3 enhanced (pandemic influenza vaccine)”**

Specifications for BSL3 enhanced (pandemic influenza vaccine) facilities include the following requirements in addition to the principles for BLS3 facilities as specified in the WHO Laboratory biosafety manual (1), and are additional to the specifications given above in section 3.4.1.

3.4.2.1 **Facility**

The facility should be designed and operated to meet the demands of protection of the recipient of the vaccine, the staff producing and testing the vaccine and of the environment. This will require specialized engineering solutions that may include:

- negative pressure secondary containment areas
- HEPA filtration on supply and exhaust air
- on-site decontamination of liquid effluent
- floor dams should be erected around bioreactors or other large scale equipment including storage tanks to contain spillage of virus from large virus-containing vessels

3.4.2.2 **Personal protection**

- All clothing worn outside the facility should be replaced by manufacturing facility garments upon entry into the facility.
- Upon entry into the containment zone personnel are to gown in full body protective single-use laboratory clothing (for example Tyvek® disposable overalls).
- When open activities are being conducted, eye protection and the use of respiratory protective equipment, such as N95, FFP3 (43) or equivalent respirators such as positive pressure air purifying respirators is required. Minimal specifications for the filtering/absorbing capacity of such equipment should be met, and masks, if used, must be fitted properly and the correctness of fit tested.