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.

Following a brief introduction, the report summarizes a number of general issues brought to the attention of the Committee. The next part of the report, of particular relevance to manufacturers and national regulatory authorities, sets out revised WHO Guidelines on the quality, safety and efficacy of candidate dengue tetravalent vaccines (live, attenuated), along with revised WHO Recommendations in relation to the production and quality control of bacille Calmette–Guerin (BCG) vaccines and of acellular pertussis vaccines. In addition, a generic protocol for the calibration of seasonal and pandemic influenza antigen working reagents is included. Revised WHO Guidelines for thromboplastins and plasma used to control oral anticoagulant therapy with vitamin K antagonists are then presented. Finally, new WHO assessment criteria for national blood regulatory systems are provided.

Subsequent sections of the report then provide information on the current status and proposed development of international reference materials in the areas of antibiotics; biotherapeutics other than blood products; blood products and related substances; in vitro diagnostic device reagents; and vaccines and related substances.

A series of annexes are then presented which include an updated list of WHO Recommendations, Guidelines and other documents on biological substances used in medicine (Annex 1), followed by a series of WHO Recommendations and Guidelines adopted on the advice of the Committee (Annexes 2–7). All additions made during the meeting to the list of International Standards and Reference Reagents for biological substances maintained by WHO are then summarized in Annex 8, and are also available at: http://www.who.int/biologicals.
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 fulfils 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.

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

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SELECTED WHO PUBLICATIONS OF RELATED INTEREST

WHO Expert Committee on Biological Standardization
Sixty-first report.
WHO Technical Report Series, No. 978, 2013 (xi + 384 pages)
web site http://www.who.int/biologicals

WHO Expert Committee on Biological Standardization
Sixtieth report.
WHO Technical Report Series, No. 977, 2013 (viii + 231 pages)

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

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

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

Further information on these and other WHO publications can be obtained from
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WHO Expert Committee on Biological Standardization

Sixty-second report

This report contains the collective views of an international group of experts and does not necessarily represent the decisions or the stated policy of the World Health Organization.
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WHO Expert Committee on Biological Standardization
17 to 21 October 2011

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

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3 For specific agenda items only – by telephone.
WHO Expert Committee on Biological Standardization  Sixty-second report

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## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Ae</td>
<td>Aedes</td>
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<tr>
<td>AE</td>
<td>adverse event</td>
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<td>AR</td>
<td>adverse reaction</td>
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<tr>
<td>BCG</td>
<td>bacille Calmette–Guérin vaccine</td>
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<tr>
<td>BRN</td>
<td>Blood Regulators Network</td>
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<tr>
<td>CBER</td>
<td>Center for Biologics Evaluation and Research</td>
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<tr>
<td>CCID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>cell culture infectious dose 50%</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
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<tr>
<td>CFU</td>
<td>colony-forming units</td>
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<tr>
<td>CMI</td>
<td>cell-mediated immunity</td>
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<tr>
<td>DENV</td>
<td>dengue virus</td>
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<td>DFI</td>
<td>dengue febrile illness</td>
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<tr>
<td>DTaP</td>
<td>acellular pertussis component DTP vaccine</td>
</tr>
<tr>
<td>DTP</td>
<td>diphtheria, tetanus and pertussis</td>
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<tr>
<td>E</td>
<td>envelope</td>
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<tr>
<td>EDQM</td>
<td>European Directorate for Quality of Medicines and HealthCare</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>ERA</td>
<td>environmental risk assessment</td>
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<tr>
<td>FDA</td>
<td>United States Food and Drug Administration</td>
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<tr>
<td>FSH</td>
<td>follicle-stimulating hormone</td>
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<tr>
<td>GCP</td>
<td>good clinical practice</td>
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<tr>
<td>GCV</td>
<td>geometric coefficient of variation</td>
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<td>GDP</td>
<td>good distribution practice</td>
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<tr>
<td>GMO</td>
<td>genetically modified organism</td>
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<tr>
<td>GMP</td>
<td>good manufacturing practices</td>
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<tr>
<td>HBsAg</td>
<td>hepatitis B surface antigen</td>
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<td>HAV</td>
<td>hepatitis A virus</td>
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<tr>
<td>HBV</td>
<td>hepatitis B virus</td>
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<td>Description</td>
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<tr>
<td>HCV</td>
<td>hepatitis C virus</td>
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<td>HEV</td>
<td>hepatitis E virus</td>
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<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
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<tr>
<td>ICDRA</td>
<td>International Conference of Drug Regulatory Authorities</td>
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<td>IPV</td>
<td>inactivated polio vaccine</td>
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<td>ISBT</td>
<td>International Society of Blood Transfusion</td>
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<td>ISTH</td>
<td>International Society on Thrombosis and Haemostasis</td>
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<tr>
<td>IU</td>
<td>International Unit</td>
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<tr>
<td>LH</td>
<td>luteinizing hormone</td>
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<tr>
<td>MHLW</td>
<td>Ministry of Health, Labour and Welfare, Japan</td>
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<tr>
<td>NAT</td>
<td>nucleic acid amplification technique</td>
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<td>NCL</td>
<td>national control laboratory</td>
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<td>NIAID</td>
<td>National Institute of Allergy and Infectious Diseases</td>
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<td>NIBSC</td>
<td>National Institute for Biological Standards and Control</td>
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<tr>
<td>NRA</td>
<td>national regulatory authority</td>
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<td>NS</td>
<td>non-structural</td>
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<td>OPV</td>
<td>oral polio vaccine</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>PDK</td>
<td>primary dog kidney</td>
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<td>PEI</td>
<td>Paul-Ehrlich-Institute</td>
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<td>prM</td>
<td>premembrane</td>
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<tr>
<td>PRNT</td>
<td>plaque-reduction neutralization test</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcription-polymerase chain reaction</td>
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<tr>
<td>QMS</td>
<td>quality management system</td>
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<tr>
<td>SOP</td>
<td>standard operating procedure</td>
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<tr>
<td>SPC</td>
<td>summary of product characteristics</td>
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<tr>
<td>SSC</td>
<td>Scientific and Standardisation Subcommittee (ISTH)</td>
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<tr>
<td>TSE</td>
<td>transmissible spongiform encephalopathy</td>
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<tr>
<td>TSH</td>
<td>thyroid-stimulating hormone</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>VE</td>
<td>vaccine efficacy</td>
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<tr>
<td>VWF</td>
<td>von Willebrand factor</td>
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<tr>
<td>VWF:Ag</td>
<td>von Willebrand factor antigen</td>
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<tr>
<td>VWFpp</td>
<td>von Willebrand factor propeptide</td>
</tr>
<tr>
<td>WHA</td>
<td>World Health Assembly</td>
</tr>
<tr>
<td>YFV</td>
<td>yellow fever virus</td>
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1. Introduction

The WHO Expert Committee on Biological Standardization met in Geneva from 17 to 21 October 2011. The meeting was opened on behalf of the Director-General by Dr Carissa Etienne, Assistant Director-General of the Health Systems and Services cluster.

Dr Etienne outlined the major issues to be discussed during the meeting. A major need in many developing countries is for guidance on the safety of blood products, especially in relation to strengthening national and regional blood regulatory systems. The Committee would also be requested to provide advice on diagnostics for Chagas disease, and on hepatitis B viral genotype diagnostics. Dr Etienne also emphasized the importance of WHO reference materials in improving and ensuring access to quality biological products. She noted that a record number of new and replacement vaccines would become available in the coming years and would be presented for adoption during future meetings, with guidelines on dengue vaccines and bacille Calmette–Guérin (BCG) vaccines, for example, being put forward for consideration at the current meeting. She reiterated that a major goal of WHO was to assure the availability of safe, effective and affordable vaccines, diagnostics, and reagents, and that this included capacity-building for regulatory authorities. Dr Etienne also told the Committee that the “Decade of Vaccines” initiative, endorsed by the World Health Assembly (WHA) in 2011, offered a unique opportunity for all involved sectors to work together to realize the full potential of vaccines in enhancing public health worldwide.

Dr Etienne concluded by reminding the members of the Committee that they served as individual experts and not as representatives of their parent organizations or countries. She also reminded them that they should participate fully in the discussions so that maximum use could be made of their expertise and that the decisions reached should be based on sound scientific considerations.

Dr Elwyn Griffiths was elected Chairman of both the overall meeting and vaccine track with Dr John Petricciani as Rapporteur, and Dr Harvey Klein was elected Chair of the blood track with Dr Anthony Hubbard and Dr Micha Nübling as Rapporteurs. The Committee then adopted the agenda (WHO/BS/2011.2161) and the timetable proposed.
2. General

2.1 Current directions

2.1.1 Strategic directions in biological standardization

Dr David Wood informed the Committee that the International Conference of Drug Regulatory Authorities (ICDRA) recommended collaboration and cooperation with WHO in several areas, including: (a) continuing to assist national regulatory authorities (NRAs) in garnering political support at national or regional levels to build regulatory capacity; (b) providing a mechanism for sharing ongoing regional harmonization activities; (c) collecting best practices for collaboration and cooperation between NRAs including information exchange, joint assessments and inspections and activities aimed at reducing duplication; and (d) facilitating the twinning of less-developed agencies with well-established agencies for capacity-building and training. NRA strengthening was one of the top 10 priorities for WHO, and a range of initiatives had been or were being developed.

The Committee was informed of the Decade of Vaccines initiative which aimed to enhance coordination across the global community by creating a global vaccine action plan (GVAP) with four working groups: (a) public and political support; (b) delivery; (c) research and development; and (d) global access. Groups b–d incorporated regulatory aspects. Priorities in the short-, middle- and long-term were to be set and a regulatory research agenda was to be developed by the end of 2011 with broad input from interested parties. The Committee noted this development and supported proposals for WHO to lead a process of regulator engagement as part of the Decade of Vaccines initiative, and to further the development of a global regulatory research agenda. Consideration may be given to the potential for extending this approach to other areas of biologicals. In that regard, the Committee recommended that WHO obtain the views of various groups and committees involved with biologicals.

The results of a survey undertaken by WHO showed that norms and standards were valued by both developed and developing countries. However, indicators were needed of the impact of WHO norms and standards in order to quantify their value to stakeholders. Survey participants also indicated that the process of priority-setting for new norms and standards should be made more transparent and inclusive. Other issues arising from the survey findings included the need for ongoing review of the continued fitness for purpose of Reference Preparations provided by WHO, and for the Committee to continue to meet at least once a year in order to provide up-to-date guidance to stakeholders. It was recognized that implementation programmes for complex standards were essential but were resource-intensive. WHO should therefore carefully evaluate the needs of stakeholders and set priorities appropriately. The need for efforts
to increase the implementation of the standards adopted by the Committee should be more clearly highlighted as part of achieving improved international harmonization.

Although there continued to be strong demand for the outcomes of WHO standardization activities, reforms were now under way to adjust the work of WHO in accordance with a new and constrained global economic reality. This would require WHO to re-evaluate its ability to maintain some of its current activities and to undertake new initiatives.

Dr Wood also informed the Committee that Sanofi Pasteur had donated type-3 polio vaccine seed strain to WHO that would be held at the National Institute for Biological Standards and Control (NIBSC), Potters Bar, Herts, England.

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

An important element of WHO biological standardization activities is to facilitate the implementation of WHO guidance in the regulation and control of product development and manufacturing. In this context, a joint meeting had been held with the International Alliance for Biological Standardization (IABS) to discuss the use of thermal stability data for vaccines. Additionally, steps were being taken to strengthen collaboration between WHO collaborating centres and NRAs. One aim was to ensure that such centres could assist countries in their respective regions, while promoting the implementation of WHO written standards and inter-laboratory collaboration. The adoption of a networking approach by WHO collaborating centres in this and other areas was seen as a potential aid to this process.

Proposals were then set out to the Committee to make draft WHO guidelines and recommendations available for public comment in order to allow revision as necessary before final presentation to the Committee for adoption. This step was intended to help speed up adoption and to facilitate the inclusion of up-to-date scientific knowledge and best practices.

WHO written standards for vaccines have evolved over the years to include technical specifications to help define safe and efficacious vaccines. These specifications are intended to be scientific and advisory in nature, and can be used as a starting point for setting national requirements and for vaccine prequalification. Proposed written standards for 2010–2013 were reviewed for a range of specific products and activities, including oral polio vaccine (OPV); combined vaccines based on diphtheria, tetanus and pertussis (DTP) vaccine; malaria vaccine; Japanese encephalitis vaccine (live, attenuated); dengue vaccine; bacille Calmette–Guérin (BCG) vaccine; acellular pertussis (aP) vaccine; inactivated polio vaccine (IPV); risk assessment activities; nonclinical evaluation; and biologicals derived by DNA technology.
Another important activity is the development of WHO guidance on the regulatory evaluation of copies of biological medicines that have come off-patent – also known as similar biotherapeutic products (SBPs) or “biosimilars” – based on sound scientific principles but without inhibiting the development and introduction of such products in a wider range of countries. A plan for the implementation of written standards in 2012–2013 covered SBPs in China, and vaccine lot releases in Latvia and India. The development of guidance on SBPs in Latin America could also usefully be considered.

A consultation on the strategic direction for standardization of vaccines and biotherapeutics was planned for 23–24 April 2012 and a first meeting of a proposed network of WHO collaborating centres was planned immediately afterwards to discuss issues such as the role of WHO and other standard-setting bodies in developing standards, and establishing networks of NRAs and national control laboratories (NCLs).

The Committee was also advised that feedback had been obtained from stakeholders concerning the biological standardization web site (http://www.who.int/biologicals) and improvements had been made as a result. Further changes would be incorporated as appropriate.

The Committee was then reminded that WHO was in the process of reform which would result in decreased resources at a time when there were increasing expectations of assistance from countries. As a result, the Committee recommended that a review of the work of the WHO Quality, Safety and Standards unit be undertaken that should include the unit business plan, the WHO constitutional mandates, country expectations, workload, impact on global public health, priority-setting, and funding sources and opportunities. The Committee noted that throughout the course of the meeting, recommendations were made for such a review of the work of the unit which it strongly endorsed.

The Committee noted the report of activities in this area and indicated its support for the proposal for WHO to provide technical support to countries on selected recently adopted written standards, and to develop impact indicators for this area of work. The Committee also recommended that WHO should consider the establishment of a process for formally numbering the different versions of written standards that were recommended by the Committee for adoption and published on the WHO web site prior to finalized publication in the WHO Technical Report Series. The Committee also recommended that in future more specific guidance should be provided by the WHO Quality, Safety and Standards unit regarding the degree of detail to be incorporated into the report of the decisions made by the Committee. The Committee considered this to be an important step in harmonizing the work of the current two meeting tracks on vaccine-related and blood-related materials.
2.1.3 **Blood products and related in vitro diagnostics: recent and planned activities in biological standardization**

The Committee was reminded that World Health Assembly resolution WHA63.12 recognized that stringent regulatory control was vital in assuring the quality and safety of blood products, and urged Member States:

*to update their national regulations…in order to ensure that regulatory control in the area of quality and safety of blood products across the entire transfusion chain meets internationally recognized standards.*

The Committee agreed that strengthening regulatory systems for blood products and building the technical capacities of national and regional blood regulatory authorities were recognized as fundamental requirements in ensuring the global availability of safe blood products.

Traceability from donor to patient was also an important component as it linked safety issues to individuals. Examples of contributions to the improved regulation of blood products were provided, including the development of several guidelines and reference materials for the control of blood products, and the identification of essential control functions and their standard indicators.

The need for stringent blood product regulation arises from: (a) the inherent risks of blood products that require an organized national or regional blood system to deal with the complexities of providing adequate, timely and equitable access to safe products; and (b) the need within such a system for a competent blood regulatory authority to ensure that appropriate production and safety monitoring standards are met.

ICDRA indicated that WHO should focus on capacity-building for the implementation of quality assurance systems for blood and blood products through the development of independent regulatory authorities. A strong focus was required on strengthening regional regulatory networks and the provision of education and training to ensure the best use of current guidelines and assessment criteria. Resolution WHA63.12 requested the Director-General:

*to ensure sustainable development and provision of International Biological Reference Preparations (International Standards) for use in the quality control and regulation of blood products and related in vitro diagnostic devices.*

In addition, WHO was requested to improve access by developing countries to WHO biological Reference Preparations and to the scientific information from collaborative studies conducted during their validation in order to ensure their appropriate use. WHO was expected to report back to the WHA by May 2014.
An often under-appreciated but essential contribution of NIBSC and the International Society on Thrombosis and Haemostasis (ISTH) lies in the procuring and supplying of the required reference materials. The Committee was informed of future plans for WHO reference materials.

The Committee indicated its support for the proposal to encourage the increased involvement of laboratories in developing countries in WHO collaborative studies of candidate global reference materials. The Committee also agreed that impact indicators should be developed for the standards it endorsed.

2.2 Reports

2.2.1 Report of the WHO Blood Regulators Network

Dr Jay Epstein, Chairman of the WHO Blood Regulators Network (BRN), presented an overview of the activities of the network which was first convened in 2006 and which addressed issues related to advancing technical expertise in the areas of blood, blood products and associated drugs and medical devices including in vitro diagnostics. The specific objectives of the network were to identify issues in the blood field, share expertise and information, promote a convergence of regulatory policy, and propose solutions to emerging public health challenges. BRN members were national authorities with comprehensive responsibility for blood regulation and the necessary expertise and capacity to address emerging public health challenges.

Since 2006, network members had provided input to WHO in the drafting of guidelines on the production, control and regulation of blood products. In addition, the exchange of information on topical issues was an ongoing BRN activity and a number of teleconferences and other events had been held. The role of the BRN had also been outlined at a number of international meetings, most recently in 2011 at the first meeting of the International Society of Blood Transfusion (ISBT) Working Party for Global Blood Safety in Lisbon, and at the 7th IABS International Symposium on Advances in Transfusion Safety in Singapore. Network members had also taken steps to ensure preparedness for the selection of blood donors in a pandemic situation, and were actively involved in the resolution of problems in the area of potency assays for recombinant products, most notably factor VIII. In November 2010, BRN assessment criteria for national blood regulatory systems had been drafted and had now undergone a process of review. An associated national system assessment tool was being developed and adoption by WHO of the BRN criteria would now be considered by the Committee. Membership of the network, for which WHO provided the Secretariat, had recently expanded from six control and regulatory authorities to seven with the inclusion of the regulatory agency of the Japanese Ministry of Health, Labour and Welfare (MHLW).
The Committee noted the developments in this area, and welcomed the inclusion of MHLW as a new member of the BRN.

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

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

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

The Committee was provided with an overview of current activities and developments at NIBSC in relation to the WHO programme for biological standardization. NIBSC had completed its studies of four replacement and six new candidate standards for adoption by the Committee. In addition, 10 project proposals were presented for endorsement by the Committee. Two stability-monitoring projects were ongoing. Other WHO-related activities included promoting vaccine quality control, developing guidelines for product development and quality control, workshop training in biological standardization and medicines control, participation in a variety of consultation and advisory committees, and the development of a variety of other standards including three haematological product standards.

NIBSC had contributed to international efforts in the fields of influenza, tuberculosis and blood products, and to collaborative projects with organizations including the Paul-Ehrlich-Institute, the Center for Biologics Evaluation and Research, and the European Directorate for the Quality of Medicines & HealthCare (EDQM).

NIBSC had also been exploring new technological developments such as non-destructive testing (O₂ monitoring and moisture content) using infrared and Raman spectroscopy. There were many potential advantages to this technique, including no wasting of material.

During the past year, approximately 20,000 vials and ampoules of reference materials had been shipped, with only a very small number of delays and other problems occurring in transit. Capacity for filling infectious materials was expected to be operational soon. However, the development and distribution of reference materials was increasingly giving rise to intellectual property issues.

The Committee was also informed that NIBSC faced a number of challenges due to the fact that the programme was already very large and there was a continuing demand for replacement standards in addition to the demand to expand activities in emerging areas such as cell standards. This was occurring at a time of severe financial pressures and decreasing government support. Although several options for identifying alternative or supplemental sources of income were being explored, current activity levels would become unsustainable in the face of decreasing financial resources.
Fourteen new and replacement reference materials from NIBSC were on the agenda of the current meeting. The preparation of all such materials was placing heavy demands on Institute resources, with increasing demands arising for work on reference materials in new areas and for new purposes, such as the provision of positive and negative control preparations and of genetic reference materials. For example, the Institute was taking on activities at national level in the field of clinical virology to improve the provision of reference materials, and this work had international implications. Progress was also being made in developing diagnostic testing for variant Creutzfeld–Jacob disease (vCJD).

A balance was thus required between replacements and new standards in established fields, and the need to support innovative products and new technologies such as viral diagnostics, genetic diagnosis and cell counting standards. Currently unresolved issues included whether the programme should expand into new areas, deciding on the most efficient and effective organizational structure, and securing adequate financial resources to carry out the mission of NIBSC. The prioritization of activities and decisions on whether to expand into new areas were matters which the Committee should consider.

The Committee noted the issues raised and recommended that WHO should consider the establishment of a review process to advise the Director-General of WHO on optimal strategies and policies to ensure that the international biological standardization endeavour remained fit for purpose and adequately resourced.

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

The PEI was originally designated as a WHO collaborating centre in 2005 and subsequently re-designated in 2009 as the WHO Collaborating Centre for Quality Assurance of Blood Products and in vitro Diagnostic Devices. The PEI was also an active BRN participant and acted as its first chair in the period 2006–2008. In addition, the Institute also collaborated with WHO in other areas, including vaccines and advanced therapy medicinal products (cell and gene therapy, and xenotransplantation), tissue repair and stem cells. The PEI had also been involved in several specific WHO projects, including work on hepatitis E, transfusion-relevant bacterial strains, a hepatitis B virus (HBV) genotype Reference Preparation for HBV DNA assays and HBsAg tests, exploring a new factor VIII potency assay, and development of a WHO International Standard for hepatitis C virus (HCV) core antigen.

PEI training courses for assessors working in regulatory agencies were conducted to support the key WHO objective of improving the regulation and control of blood products. This was in line with resolution WHA58.13 and report EB113/10, and with resolution WHA63.12. Because of its extensive experience in
the regulation of in vitro devices and blood products, the PEI assessor training programme was used by regulatory authorities and agencies worldwide.

Other collaborative activities with WHO had included work on the WHO prequalification programme for in vitro diagnostics. Starting in 2009, recent collaborations had focused on the prequalification of rapid assays for the detection of HIV and malaria with PEI involvement in dossier reviews; site inspections; development of guidance for reviews; provision of suggestions for fast tracking product dossiers; and the preparation and organization of a workshop on HIV nucleic acid amplification techniques (NATs).

Recent collaborative activities with other WHO collaborating centres included the Third Meeting of WHO Collaborating Centres for Biological Standards to support the development of WHO biological Reference Preparations for blood products and in vitro diagnostic devices, and participation in collaborative studies of WHO international blood product standards.

**Center for Biologics Evaluation and Research (CBER), Bethesda, MD, USA**

The Committee was provided with a brief overview of the regulatory responsibilities of CBER – which did not include diagnostics or certain therapeutic biologicals – and its attention was drawn to a number of recent organizational changes. In its work, CBER interacted with a range of governmental and nongovernmental organizations, and adopted a strategic approach to reference materials involving the external accreditation of producers.

Recent CBER activities in the field of vaccines included the successful negotiation of a CBER-WHO Cooperative Agreement with substantial funding provided by the United States Food and Drug Administration (FDA) to WHO with the potential for further funding for up to five years. This FDA and WHO collaboration aimed to support scientific collaboration and enhance the regulatory capabilities of NRAs in order to advance global access to safe and effective vaccines and other biologicals that meet international standards. In its first year the programme would focus on enhancing regulatory capacity for seasonal and pandemic influenza vaccines. Strengthening regulatory capacity would bring wider benefits in terms of vaccine access and global supply.

Other recent activities included meetings and training on the regulation of biologicals, and the introduction of a publicly available web-based programme intended to provide information on the regulatory work carried out at CBER.

CBER had also collaborated closely with a number of organizations particularly in the area of influenza preparedness, including continued collaboration with WHO and other partners in strengthening vaccine virus strain selection and reagent preparation for seasonal influenza vaccines, and in the development of candidate vaccine strains for viruses with pandemic potential.
Other activities included work on developing a process (“animal rule”) to allow for the licensing of vaccines when human efficacy studies were not ethical or feasible. Although no vaccines had yet been licensed using the animal rule, potential applications included the use of anthrax vaccines for post-exposure prophylaxis and new-generation smallpox vaccines.

The Committee was also informed of CBER-sponsored workshops and research activities conducted or scheduled for 2011–2012. These included one workshop on the development and evaluation of next-generation smallpox vaccines, and one on the development and evaluation of human cytomegalovirus vaccines. Selected research projects were also initiated to develop and evaluate novel methods that enhance the safety of vaccine-related products, and to advance the use of the animal rule for efficacy evaluation of vaccines and facilitate pre-clinical evaluation.

2.3 Issues

2.3.1 Scientific issues identified by users of WHO biological Reference Preparations

The Committee was informed of the links that existed between the EDQM and WHO. The EDQM was responsible for producing the European Pharmacopoeia and, specifically in the biological field, for European reference materials which were calibrated against WHO primary reference materials. The EDQM was also responsible for a programme of method development and standardization overseen by a Steering Group on which WHO sat. The EDQM was also the custodian of the remaining WHO antibiotic reference materials.

The Committee was informed that 114 projects had been initiated or concluded – 36 on method development and 78 on reference materials. Some of the projects involved collaboration with WHO (for example, replacement of the tetanus vaccine standard and of the endotoxin standard replacement, both of which were ongoing). Other ongoing projects of potential interest to WHO included: (a) alternative assays for testing for pertussis toxin, with the goal of validation and introduction of the best method into the European Pharmacopoeia; (b) pertussis vaccine (acellular) potency assay improvement; (c) pertussis vaccine (whole cell) potency assay improvement; (d) veterinary use of rabies vaccine involving the replacement of the mouse potency test that may have implications for the human rabies vaccine assay; and (e) recombinant major allergens calibrated in SI units.

As part of a European Union commitment to develop alternatives to animal testing, WHO was invited to send a representative to participate in ongoing efforts to refine, reduce and replace the use of animal testing methods in the quality control of biologicals. Five projects on improving testing methods...
included three that were intended to replace in vivo with in vitro techniques, and may have a significant impact on the published WHO recommendations for quality control of the products involved.

The Committee was also informed that there were many potential new areas of work in the biologicals field such as cell and tissue therapy. As a result, it was difficult to determine which new areas should be pursued. One potential mechanism for assisting WHO in this activity would be to establish an external review of the WHO biological standardization programme that would include an assessment of opportunities in new areas.

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

**Request from the WHO Immunization Practices Advisory Committee (IPAC) to establish harmonized standards for the labelling of vaccines**

The Committee was informed about the role of IPAC which was established in 2010 to advise WHO on issues related to immunization practices as part of efforts to strengthen and improve the delivery of immunization programmes at country level. In this capacity the attention of the Committee was brought to a number of labelling legibility and harmonization issues identified through an open process by the Vaccine Presentation and Packaging Advisory Group (VPPAG), which was established in 2007 by the GAVI Alliance. These issues included text legibility, minimum requirements, multiple language requirements, package insert information, use of generic names on labels, ability to observe vial content by allowing a minimal clear area and the date format. The Committee was reminded that it had responsibility for good manufacturing practice (GMP) for biologicals and standards for specific products such as vaccines. The Committee was requested to review these issues and to consider possible solutions to the issues raised.

The Committee noted the report and agreed with a proposal that the WHO Secretariat should explore two options for the harmonization of labelling standards for vaccines, namely (a) to prepare a revision of section 7 (labelling) of the current WHO GMP for biologicals; or (b) to investigate the promotion of a new standard through tender specifications for United Nations procurement.

**Proposed Third WHO International Standard for bacterial endotoxin**

There was agreement that the only reliable approach to maintaining harmonization of the endotoxin unit for pyrogen testing was through the establishment of a shared and harmonized reference material. Current procedures for endotoxin testing were fully harmonized between the European, Japanese and United States pharmacopoeias. However, the Committee was informed that the current Second
WHO International Standard for endotoxin established in 1996 now needed to be replaced because it was nearly depleted (WHO/BS/2011.2182). The production of 75,000 vials of a candidate Third WHO International Standard was being completed. Following filling at NIBSC in November 2010, the United States Pharmacopeial Convention (USP), NIBSC, and EDQM sponsored a collaborative evaluation study involving 33 laboratories worldwide. The results of the study would be submitted to the Committee for consideration in 2012 and the replacement International Standard would be proposed.

The Committee endorsed the project as proposed.

Proposed global legally binding instrument on mercury: implications for pharmaceuticals

The Committee was informed that there was growing concern about the human health implications of mercury, especially in children who receive mercury in vaccines. Thiomersal is widely used in vaccines in both developed and developing countries. Data suggested that such vaccines were very safe. In addition, alternative acceptable preservatives were not available, and the use of small-dose vials would probably be cost prohibitive and have adverse environmental impacts. WHO continued to recommend multi-dose vaccine vials for routine immunization programmes in many countries because they are safe and effective, they limit the required storage capacity and help reduce vaccine costs.

Other potential sources of mercury exposure include dental amalgam, thermometers and blood pressure measuring devices. In all of these contexts there had been a general trend to reduce and ultimately eliminate mercury exposure. However, almost all countries still use dental amalgam and alternatives are more expensive and have technical limitations. At a 2009 technical meeting co-supported by the United Nations Environment Programme (UNEP), WHO had recommended that a phase-down be pursued by promoting disease prevention and alternatives to amalgam; conducting research and developing cost–effective alternatives; educating dental professionals; and raising public awareness.

At the upcoming third session of the Intergovernmental Negotiating Committee to prepare a global legally binding instrument on Mercury (INC3) from 31 October to 4 November 2011, the use of mercury in pharmaceuticals would be considered. WHO participates in the INC process with observer status. As well as being present at the INC sessions, WHO provided submissions, technical briefings, and contributions to Secretariat meeting documents. The aim of WHO participation in the treaty discussions would be to exclude vaccines containing mercury from being prohibited, by excluding them from the scope of the treaty so as to allow their continued availability in multi-dose vials that provide safe and effective prevention of serious diseases worldwide.

The Committee noted the report.
3. International Recommendations, Guidelines and other matters related to the manufacture and quality control of biologicals

All Recommendations and Guidelines established at the meeting are listed in Annex 1, which provides an updated listing of all current WHO Recommendations, Guidelines and other documents related to the manufacture and quality control of biological substances used in medicine.

3.1 Vaccines and related substances

3.1.1 Guidelines on the quality, safety and efficacy of dengue tetravalent vaccines (live, attenuated)

Dengue is a mosquito-borne disease that represents a major public health problem in tropical regions of the world. The draft Guidelines (WHO/BS/2011.2159) that were considered by the Committee covered dengue tetravalent vaccines (live, attenuated) under clinical development, and were intended to provide guidance to NRAs and vaccine manufacturers on their quality, safety and efficacy to facilitate their international licensure and use.

Other types of dengue virus vaccines under development (such as subunit or inactivated vaccines) were outside the scope of these Guidelines. However, some of the guiding principles provided – for example, in Part C on clinical evaluation – may be useful for the evaluation of these other vaccine types. In addition, in terms of quality-control aspects, the guiding principles applicable to subunit, inactivated or other vaccine types were available elsewhere if the product in development shared a similar manufacturing process. For example, guidelines for human papillomavirus and hepatitis B vaccines may also be useful for subunit dengue vaccines.

The draft Guidelines had been based on the experience gained from developing candidate tetravalent dengue vaccines (live, attenuated), and would need to be updated as new data became available from additional studies. Following a brief overview of dengue disease and dengue vaccine development, Part A sets out the guidelines for manufacture and quality control, with the specific issues of nonclinical evaluation, clinical evaluation and environmental risk assessment covered in Parts B, C and D respectively. Part E then provides guidelines for NRAs.

After making suitable amendments, the Committee recommended that the Guidelines be adopted and appended to its report (Annex 2).

3.1.2 Recommendations to assure the quality, safety and efficacy of BCG vaccines

Tuberculosis was declared a global emergency by WHO in 1993, and Mycobacterium tuberculosis (M. tuberculosis) was now considered to be responsible
for more adult deaths than any other pathogen. Vaccination with the BCG vaccine remained the standard for tuberculosis prevention in most countries because of its efficacy in preventing life-threatening forms of the disease in infants and young children. It was inexpensive and usually required only one administration in either neonates or adolescents. As there was currently no suitable alternative, BCG would remain in use in the foreseeable future and may continue to be used as a prime vaccine in a prime-boost immunization schedule in conjunction with new tuberculosis vaccines.

The last revision of the Requirements for BCG vaccine for human use was in 1985, with an amendment updating the section on the expiry date published in 1998. Recent WHO consultations had addressed the issues of improving BCG vaccine characterization and quality-control assays to reflect current state-of-the-art technology. In addition, a proposal had been made to replace the WHO International Reference Preparation for BCG vaccine by substrain specific Reference Reagents evaluated by collaborative studies. The draft Recommendations (WHO/BS/2011.2157) that were considered by the Committee covered the production and control of BCG vaccines in Part A, with guidelines for nonclinical evaluation provided in Part B. Guidelines on the content of the clinical development programme applicable to BCG vaccines were provided in Part C, and recommendations for NRAs in Part D.

The nonclinical evaluation guidelines applied to classical BCG vaccine products still in need of such evaluation, including newly manufactured products requiring clinical trial studies or in case of manufacturing changes.

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

3.1.3 **Recommendations to assure the quality, safety and efficacy of acellular pertussis vaccines**

Pertussis immunization is an integral part of immunization programmes in all regions of the world. It is recommended for all infants and children and in some countries also for adults and adolescents. Whole-cell pertussis vaccines have been used for more than 50 years, have been shown to provide protection and are still the foundation of global pertussis control. However, there is increasing interest in acellular pertussis vaccines, which have also been shown to be safe and effective and which have been successfully introduced into many national immunization programmes.

As a consequence of increasing demand for acellular pertussis vaccines, new manufacturers are entering the field. The expansion in the number and use of acellular pertussis vaccines, the development of new vaccines and advances in the standardization of quality-control methods have prompted WHO to update the current WHO Guidelines for the production and control of the acellular pertussis component of monovalent or combined vaccines.
The Committee was informed that the current Requirements for acellular pertussis vaccine were adopted in 1996 with the recognition that further improvements in their production and evaluation would follow. Since 1996, further experience had been gained and it had become clear that a revision should be undertaken. Acellular pertussis vaccines were almost exclusively administered in combination with diphtheria and tetanus toxoid vaccines. Moreover, in recent years, there had been increased interest in the use of more complex combination vaccines, a trend that increased the challenges of clinical evaluation. Furthermore, evaluating the clinical efficacy of new acellular pertussis vaccine formulations had become increasingly difficult due to the decreased prevalence of pertussis cases worldwide.

The draft revised Recommendations (WHO/BS2011.2158) that were considered by the Committee highlighted advances in the development, manufacturing and testing of acellular pertussis vaccines and aimed to provide guidance on: (a) improving the quality control of existing vaccines based on new information and experience; and (b) evaluating new products and new combinations through manufacturing control and through both nonclinical and clinical studies. The parameters measured during testing should include immunogenicity and safety, and the candidate vaccine should be shown to be non-inferior to the licensed comparator vaccine. After making suitable amendments, the Committee recommended that the Recommendations be adopted and appended to its report (Annex 4).

3.1.4 Assessing risk when a potential adventitious agent is found

The Committee was reminded that in 2010 it had recommended that WHO lead the development of a risk assessment process subsequent to marketing authorization. In addition, the ICDRA had recommended that WHO assist countries in developing risk management strategies for responding to scientific advances in detecting adventitious agents in biological products.

Following the discovery of porcine circovirus (PCV-1,2) in rotavirus vaccines in 2010, NRAs and manufacturers assessed the emerging data on an ongoing basis and made decisions accordingly, in line with previous discoveries of an adventitious agent in licensed biological products. WHO was requested to develop a document on assessing risk in such circumstances, and a first draft version was considered by the Committee in 2010, who requested that it be revised after broad consultation. Since then, several meetings had been held to discuss the revised document which was now in the process of further revision with an expectation that a final document would be submitted to the Committee in 2012 for adoption.

A progress report was presented to the Committee who then provided a number of suggestions to be considered during the revision process, while
emphasizing the importance of coordination and collaboration among WHO, regulatory agencies and manufacturers whenever a potential adventitious agent was identified.

3.1.5 Calibration of seasonal and pandemic influenza antigen working reagents by WHO essential regulatory laboratories

The Committee was informed of the process used to develop a generic protocol for the calibration of seasonal and pandemic influenza antigen working reagents by WHO essential regulatory laboratories (ERLs) and a draft generic protocol was provided for the Committee to review (WHO/BS/2011.2183). The Committee expressed its appreciation for the work that had gone into the development of both the process and the generic protocol.

Reagents for assessing the potency of influenza vaccines using the single radial immunodiffusion (SRID) assay are prepared annually as the strains in the vaccine are updated. The calibration process involves the preparation of a primary liquid standard (PLS) and a large batch of freeze-dried antigen by one of the ERLs. The PLS is then distributed to all the other ERLs for independent calibration. Samples of the freeze-dried antigen are also distributed to the other ERLs and calibrated against the PLS using the SRID assay.

Four ERLs are involved in this activity which had hitherto been outside the Committee process because of the intense time constraints involved. To make the process more transparent, the generic protocol provided described in detail the process by which the four ERLs developed and calibrated the reagents. After making suitable amendments, the Committee recommended that the generic protocol be adopted, appended to its report (Annex 5) and published on the WHO web site.

3.1.6 Proposed new projects for developing or updating written standards

The Committee was informed that the WHO Prequalification unit had established a priority list (high, medium, and low) for vaccines to be used in global immunization programmes. The Secretariat also had a list of new and replacement regulatory written standards for vaccines and other biologicals. The Committee was further informed that 12 new vaccines were in various stages of development and were expected to be introduced by 2021. Of those 12, seven were improvements on existing vaccines, and five were new vaccines. The Committee was requested to assist the WHO Quality, Safety and Standards unit by providing guidance on identifying the criteria that should be used in prioritizing those biologicals for which written standards should be developed.

After a full discussion, the Committee recommended that the unit consider the following factors: (a) public health emergencies are likely to arise from time to time and will of necessity become a top priority; (b) the global public
health importance of a specific standard; (c) the vaccines assigned high priority by the WHO Prequalification unit; (d) the establishing of high-, medium- and low-priority categories within the unit based on input from stakeholders (such as Member States through WHO regional offices, international organizations, the Committee, regulatory agencies and industry organizations); (e) platform technology documents; (f) the availability of guidance documents from other organizations and whether a WHO document on the same topic added sufficient value to warrant its production; (g) building on currently available documents in new areas such as cell and gene therapy; (h) assessment of the availability of funding for new document development; (i) the occasional inclusion of several low-priority items as resources permit; and (j) the needs of countries and regions for guidance on non-vaccine products such as SBPs and biotherapeutics. The Committee suggested that the most efficient way to obtain input on priorities from stakeholders would be to request comments on a draft based on the factors listed above. The Committee also noted that a balance should be struck between revisions of existing documents and the development of new documents. Another factor that must be taken into consideration was the implementation of written standards. All such activities were directly related to NRA strengthening and would require financial resources.

The Committee welcomed the initiative for establishing a procedure for priority-setting, and it was agreed that the vaccine track Secretariat would follow up on the Committee’s suggestions, update the one-page outlines of proposed projects and provide subsequent feedback.

3.2 Blood products and related substances

3.2.1 Guidelines for thromboplastins and plasma used to control oral anticoagulant therapy with vitamin K antagonists

The draft Guidelines (WHO/BS/2011.2165) that were considered by the Committee were a revision of the previous Guidelines introduced in 1999, and described the establishment and maintenance of the International Normalized Ratio (INR)/International Sensitivity Index (ISI) system used to control oral anticoagulant therapy, and the development of the International Reference Preparations for thromboplastins. The Guidelines covered the ISI calibration of secondary standards (Procedure 1), individual batches of thromboplastin reagents (Procedure 2) and local system calibration (Procedure 3).

It was suggested that there should be a better separation between the sections relevant to manufacturers and those relevant to clinical laboratories. However, it was considered extremely difficult to separate these aspects. Manufacturers were expected to be aware of the whole document and to be responsible for instructing clinical laboratories on the use of products. It was agreed that a paragraph be added to the beginning which addressed how the document should be used and its relevance to manufacturers and clinical laboratories.
The draft Guidelines incorporated references to the SSC/ISTH Guidelines on the preparation, certification and use of certified plasmas for ISI calibration and INR determination which were published in 2004. There was still a requirement to use the manual technique with the current WHO International Standard and some concern was expressed over the maintenance of expertise when most laboratories used automated methods. However, the performance of more than 20 participating laboratories in the calibration of the WHO International Standard rTF/09 indicated that the required expertise was still present. It was announced that WHO would be willing to support workshops to maintain expertise in the manual tilt tube technique.

The possibility of replacing the manual technique (used with the International Standard thromboplastins in Procedure 1) with an automated procedure which has been validated and found to correlate with the manual technique was also discussed. Information on suitable automated procedures could be obtained through a collaborative study and included later in an appendix. It was agreed that the opinion of the SSC/ISTH Subcommittee on Control of Anticoagulation would be sought to see if this alternative would be useful.

It was agreed that a new section addressing the criteria to apply in the choice of thromboplastin reagents should also be added, possibly as an appendix.

The Committee recommended that the revised Guidelines be adopted and appended to its report (Annex 6).

### 3.2.2 Assessment criteria for national blood regulatory systems

These assessment criteria for national blood regulatory systems (WHO/BS/2011.2174; QAS/2011.431) were the result of a BRN initiative which aimed to promote robust NRAs in both developed and developing countries.

The tool consisted of an assessment framework to highlight the strengths of NRA programmes for the regulation of blood, blood components, plasma-derived products, and associated substances and medical devices including in vitro diagnostics. The assessment criteria were designed to identify gaps for future development and thus strengthening of the regulatory oversight of NRAs. They were structured into two essential elements (national regulatory system and national regulatory authority) and 12 core functions. The main criteria were broken down into indicators which were then divided into two categories – required (R) or desirable/suggested (S) – in order to accord with international best practices for national blood regulatory systems. This tool would support NRA establishment and capacity-building and promote regulatory convergence. Using previous versions of this tool, Health Canada, Swissmedic and two Latin American countries had undertaken self-assessments. Based on the experience gained, modifications had then been introduced into the document. Further input had been obtained at the ICDRA workshop in Singapore in December...
2010. A recent broad consultation process then generated comments from all six WHO regions, involving 40 countries, which had been reviewed by the BRN.

It was expected that the tool would help to identify gaps and main priorities based upon which capacity-building programmes could be developed to support the introduction of regulations for blood products at global level, and to sustain implementation of resolution WHA63.12 on the availability, quality and safety of blood products.

The Committee highlighted a need for WHO to develop an implementation plan, including a guidance document on the correct use of the tool, as part of the capacity-building activities requested in the above resolution.

After making suitable amendments, the Committee recommended that the assessment criteria be adopted and appended to its report (Annex 7).
4. International reference materials – vaccines and related substances

All reference materials established at the meeting are listed in Annex 8.

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

4.1.1 First WHO International Standard for meningococcal serogroup C polysaccharide

Meningococcal serogroup C (MenC) plain polysaccharide (PS) and conjugate vaccines are primarily evaluated by physicochemical methods to ensure that batches are consistently manufactured. As different assays are employed to quantify the MenC PS content of final formulations and bulk intermediaries, there was a need for an International MenC PS Standard to calibrate internal references used in the different laboratories. Twelve laboratories from nine different countries participated in a collaborative study (WHO/BS/2011.2169) to assess the suitability and determine the MenC PS content of a candidate International Standard MenC PS preparation (08/214).

The Committee recommended the adoption of preparation 08/214 as the First WHO International Standard for meningococcal serogroup C polysaccharide, with a content of 1.192 mg MenC PS/ampoule.

4.1.2 First WHO International Standard for anti-pneumococcal antibodies in serum

To develop and evaluate the efficacy of pneumococcal vaccines it was important to have an accurate method for measuring the concentration of human antibodies directed against pneumococcal capsular polysaccharides (Pn PS). In 2000, representatives from academia, government and industry met at WHO and selected an enzyme-linked immunosorbent assay protocol for the quantification of human IgG antibodies specific for Streptococcus pneumoniae capsular polysaccharides (Pn PS ELISA). In order to estimate the concentration of antibodies, the reference serum 89SF was produced and antibody concentrations established. As quantities of 89SF were now approaching depletion, a replacement (007sp) had been produced in order to maintain the link to clinical efficacy. Five laboratories from three countries had participated in a collaborative study (WHO/BS/2011.2164) to assess the suitability and determine the pneumococcal serotype specific antibody content of the candidate 007sp International Standard serum preparation.

The Committee recommended the adoption of preparation 007sp as the First WHO International Standard for anti-pneumococcal antibodies in serum.
The Committee noted that μg/ml was used in order to maintain continuity with 89SF which was linked to clinical efficacy. The proposed WHO International Standard was being held in two custodian laboratories: NIBSC (5000 vials) and CBER (10 000 vials) and was being stored at −20 °C. Stability data during shipping at room temperature were requested by the Committee.

4.1.3 Third WHO International Standard for trivalent inactivated polio vaccine

The potency of inactivated polio vaccine (IPV) is measured in vitro using a validated ELISA test with suitable Reference Preparations and is expressed in D-Antigen units. The Second WHO International Standard for IPV (NIBSC Code: 91/574) was established in 1994. It was shown to be suitable for determining the antigenic content and immunogenicity of IPVs by in vitro and in vivo assays respectively. In anticipation of 91/574 stock depletion, a collaborative study had been conducted in 2009 to assess the suitability of candidate replacements. However, for reasons which were not clear, this study had demonstrated excessive variability in potency estimates for the candidate standards between laboratories. Following the suggestion that this may have been due to a lack of homogeneity between vials of the candidate standards, a further pilot study had been conducted in 2010 (WHO/BS/2011.2162). A subset of six laboratories that had participated in the original study was selected to be representative of the range of potency estimates observed in the original study.

The aims of the 2010 study were to see if the variability observed between laboratories in the original study could be confirmed, and to assess the contribution of any vial-to-vial variability to the outcome.

The Committee was informed that the study had confirmed that variability between laboratories was an issue. A new collaborative study was planned for early 2012 using samples from various IPV manufacturers that would be filled at facilities other than NIBSC.

4.2 Proposed new projects – vaccines and related substances

4.2.1 Replacement of the First WHO International Standard for antibody to influenza A virus subtype H1N1pdm

To facilitate and improve transparency in the priority-setting process for new projects, a simple tool had been developed that described their salient features. The resulting documentation provided a means for the Committee and other stakeholders to review and comment on proposed new projects that were under consideration. Among the proposals made in document WHO/BS/11.2177 (Appendix 1) was the development of the Second WHO International Standard for antibody to influenza A virus subtype H1N1pdm.
Influenza is considered to be of considerable public health importance in most countries, and the H1N1pdm virus continues to circulate globally. The variability of serology assays means that it is very difficult to compare different vaccines, with resulting scientific and regulatory challenges. Nevertheless, H1N1pdm virus antigen is a component of numerous influenza vaccines which are licensed and in use.

Stock levels of the current International Standard were low and were not expected to be sufficient for future levels of demand. Anticipated uses of a replacement Reference Preparation include calibration of serology assays for vaccine trials and serum surveys with users ranging from NCLs, independent serology laboratories, clinical laboratories and vaccine manufacturers. Issues raised by this proposal included the need to determine whether the new candidate International Standard would reduce assay variability to a similar extent to the current standard and whether the assigned unitage would be similar.

The Committee endorsed the proposal to develop the Second WHO International Standard for antibody to influenza A virus subtype H1N1pdm, and urged that efforts be made to match the characteristics of the source material used for the current International Standard.

4.3 Ongoing stability monitoring – vaccines and related substances

4.3.1 Inactivated hepatitis A vaccine

The First WHO International Standard for inactivated hepatitis A vaccine was established in 1999 and has been widely used without problem. However, a loss of potency (complete loss of antigenic activity) was reported in one laboratory. This was investigated and attributed to the presence of a low-level bacterial contaminant in a small percentage of samples. For this reason a post-establishment stability evaluation was conducted (WHO/BS/2011.2160) of the standard preparation (95/500).

In addition, although stability samples were laid down at the time of production no evaluation of stability was performed for the establishment report. At its meeting in 1999, the Committee had recommended ongoing stability monitoring as the preparation was in liquid form and predictions of its long-term stability were not available because of the inherent variability of the assays. The current stability evaluation only included data on vaccine antigen.

Samples were tested for antigen content by comparison with frozen baselines stored continuously at −150 °C. No evidence was found of significant loss of stability over the 16 years of sample storage at −70 °C. Although extended studies confirmed an appropriate level of stability for use, it was proposed that this International Standard be replaced as soon as possible given the small percentage of vials that were found to be contaminated. The Committee was informed, however, that vials not contaminated were stable and fit for purpose.
The Committee recommended that information on potential contamination be included with future shipments of the current standard.

4.4 Progress reports – vaccines and related substances

4.4.1 Characteristics of an improved potency assay for inactivated influenza vaccines

The Committee was informed of a draft document on the proposed characteristics of an improved potency assay for inactivated influenza vaccines. The Committee expressed appreciation for the efforts involved in developing the draft, and encouraged further discussion with appropriate groups.

4.4.2 Hepatitis B vaccine

The Committee was informed of interest from laboratories around the world in developing a standardized assay for assessing the potency of hepatitis B vaccines based on a protocol developed by a manufacturer. A feasibility study had been undertaken by three NCLs to evaluate the impact of using different reagents on the proposed standard assay. Initial results had been encouraging and indicated that the assay could be used with a variety of different reagents. Further discussion was now needed on whether a larger collaborative study should be undertaken. The Committee requested that it be kept informed of progress.
5. International reference materials – blood products and related substances

All reference materials established at the meeting are listed in Annex 8.

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

5.1.1 Assignment of a value for von Willebrand factor propeptide to the Sixth WHO International Standard for factor VIII/VWF plasma

The ratio of von Willebrand factor propeptide to von Willebrand factor antigen (VWFpp/VWF:Ag) is used to identify bleeding disorders associated with a decreased half-life of VWF in the circulation. Since there was no internationally agreed reference material or International Unitage (IU) for VWFpp, laboratories have had to rely on locally calibrated reference materials, leading to increased inter-laboratory variability. The objective of this project (WHO/BS/2011.2171) was to assign a value for VWFpp to the Sixth WHO International Standard for factor VIII/VWF plasma (07/316) in order to define the IU and provide long-term continuity for the calibration of all secondary standards. As part of a collaborative study, 13 laboratories had estimated VWFpp (and VWF:Ag) in the International Standard relative to their local reference materials. The inter-laboratory variability for VWFpp estimates was surprisingly low (GCV 8.1%) and this provided an opportunity to assign a consensus mean value of 1.03 IU/ampoule to the International Standard. When estimates of VWFpp in a second lyophilized plasma were calculated using the International Standard as standard, the inter-laboratory variability was further reduced to a GCV of 3.0%, indicating that a VWFpp standard should lead to improved agreement between laboratories. It was noted that estimates of VWF:Ag in the International Standard, relative to local reference materials, were more variable (GCV 12.2%) than estimates of VWFpp (GCV 8.1%) and this probably indicates inaccurate calibration of local VWF:Ag Reference Preparations. It was proposed that the Sixth WHO International Standard for factor VIII/VWF plasma (07/316) be assigned a value of 1.03 IU/ampoule for VWFpp.

The Committee recommended the adoption of this assigned value.

5.1.2 Third WHO International Standard for fibrinogen (plasma)

The International Standard for fibrinogen (plasma) was introduced to improve agreement between laboratories measuring plasma fibrinogen levels in connection with the diagnosis of deficiency or in relation to the risk of cardiovascular disease. Declining stocks of the Second WHO International Standard for fibrinogen
International reference materials – blood products and related substances

A collaborative study (WHO/BS/2011.68) involving 21 laboratories from 11 countries was undertaken to assign a value for clottable protein to the proposed Third WHO International Standard for fibrinogen (plasma) (09/264) by assay relative to the current International Standard. Value assignment was based on a combination of results from Clauss assays (22 estimates) and clot-removal methods (two estimates) which provided a consensus mean value of 2.67 mg/ampoule \( (n=24) \) with inter-laboratory variability (expressed as GCV) of 3.3%. Results from immunological methods were not included in the calculated overall mean value. Stability had been assessed by an accelerated degradation study which had predicted a loss of 0.004% per year for ampoules stored at 20 °C.

The Committee recommended the adoption of 09/264 as the Third WHO International Standard for fibrinogen (plasma) with an assigned value of 2.7 mg per ampoule.

5.1.3 First WHO International Reference Reagent for anti-human neutrophil antigen-1a antibody

Transfusion-related acute lung injury (TRALI) is a recognized cause of mortality and morbidity following blood transfusion. TRALI can be caused by antibodies in the donor plasma directed against human leukocyte antigen (HLA) or human neutrophil antigen (HNA) in the recipient. Many laboratories now test for neutrophil antibodies but there are no reference reagents to determine detection sensitivity. A pilot study of a candidate lyophilized reference plasma preparation (09/284), containing a clinically relevant anti-human neutrophil antigen (anti-HNA-1a) antibody confirmed the absence of other HNA and HLA antibodies and indicated a suitable titre range. Furthermore, the study indicated the unsuitability of using one monoclonal antibody (3G8) as capture antibody for anti-HNA-1a detection in the MAIPA assay. A subsequent collaborative study (WHO/BS/2011.2167) involving 24 laboratories returned titres (highest dilution giving a positive result) ranging from 1 in 2 to 1 in 256. This wide range indicated the need for improved sensitivity in some laboratories. There was overall agreement that the minimum sensitivity required should be set at a dilution of 1 in 4. It was proposed that the preparation 09/284 be established as the First WHO International Reference Reagent for anti-human neutrophil antigen-1a (minimum potency). It was noted that antibodies to other human neutrophil antigens were also of clinical significance and could be addressed in future Reference Preparations.

The Committee recommended the adoption of 09/284 as the First WHO International Reference Reagent for anti-human neutrophil antigen-1a antibody.
5.1.4 First WHO International Reference Reagents for blood group genotyping

Blood group genotyping is increasingly being used as it has a number of advantages over conventional blood group serology. However, ISBT workshops have demonstrated a need for DNA reference materials to improve diagnostic accuracy. Four lyophilized genomic DNA preparations from genotyped and phenotyped donors were evaluated for their suitability to serve as International Reference Reagents for common Caucasian (samples RBC1, RBC4 and RBC5) and black African (sample RBC12) alleles in an international collaborative study involving 29 laboratories from 19 countries (WHO/BS/2011.2166). Although the study demonstrated an overall high level of accuracy in blood group genotyping for common alleles, the identification of errors and inconsistencies, and the limited genotyping capabilities of many laboratories, highlighted a need for validated reference materials to control test procedures and ensure that they are sufficiently sensitive. The finding that the majority of laboratories reported genotypes in accordance with those determined by the study organizers and with the serological phenotypes validated the use of RBC1, RBC4, RBC5 and RBC12 as Reference Reagents for blood group genotyping. Accelerated degradation studies indicated that the preparations would be stable at –20 °C for many years. These Reference Reagents were intended for qualitative use only – i.e. to give positive or negative results for a particular allele from which red blood cell phenotype could be inferred; the quantification of DNA was not required. It was proposed that RBC1 (10/232), RBC4 (10/236), RBC5 (10/238) and RBC12 (10/234) be collectively established as the First WHO International Reference Reagents for blood group genotyping to control test procedures for common alleles in Caucasian and black African populations. It was anticipated that up to four more preparations may be developed in the future to cover all major clinically important blood group genotypes.

The Committee recommended the adoption of the four preparations RBC1 (10/232), RBC4 (10/236), RBC5 (10/238) and RBC12 (10/234) as the First WHO International Reference Reagents for blood group genotyping.

5.1.5 Revision of the instructions for use of the First WHO International Reference Repository for platelet transfusion relevant bacterial strains

Both bacterial-screening and pathogen-reduction systems have been developed to address the bacterial contamination of platelet concentrates which remains a significant problem in transfusion medicine. A four-member panel consisting of deep-frozen bacterial strains of different bacterial species (*Staphylococcus epidermidis*, *Klebsiella pneumoniae*, *Streptococcus pyogenes* and *Escherichia coli*) able to grow in platelet concentrates after low-count spiking was assessed in a worldwide validation study.
In 2010, this panel was adopted by the Committee as the First WHO International Reference Repository for platelet transfusion relevant bacterial strains. The proposed future expansion of the repository was also endorsed.

Enlargement studies using a revised protocol were in progress and would be presented to the Committee in due course. These studies involved representative laboratories from additional WHO regions and would incorporate the validation of bacterial detection after the direct inoculation of platelets.

The instructions for use for the current repository bacterial strains were presented and questions raised by the Committee and by customer laboratories were discussed. It was agreed that on the basis of current data a number of modifications to the instructions for use should be made including: (a) a restriction on “Intended Use” for bacterial detection methods until suitability for pathogen-inactivation methods had been demonstrated; (b) the inclusion of a handling and dilution protocol; (c) the definition of precautionary measures to be followed by users; (d) potential offer of training in panel use; and (e) the removal or revision of inadequacies in phrasing and comprehensibility. In the meantime, modifications to the instructions for use had been submitted by Dr Thomas Montag-Lessing (PEI).

The Committee accepted the revised instructions for use, subject to final edits.

5.1.6 Apolipoprotein B

There is increasing evidence that elevated serum apolipoprotein B concentration is an important risk factor for coronary heart disease, and many groups recommend the measurement of this analyte over low-density lipoprotein (LDL) cholesterol. The standardization of apolipoprotein B assays has been achieved through use of the First WHO International Reference Reagent for Apolipoprotein B (SP3-07) which is a frozen pooled human serum preparation stored at the Centers for Disease Control and Prevention (CDC), Atlanta. As stocks of SP3-07 were approaching depletion, a replacement (SP3-08) had been prepared which also consisted of a frozen pooled human serum preparation. Value assignment of SP3-08 was performed relative to SP3-07 in one laboratory using an immunonephelometric method. A mean value of 1.18 g/l from 186 determinations with a CV of 2.4% was assigned. Evaluation studies had indicated that SP3-08 was suitable for use in terms of stability, linearity of dilutions and the calibration of manufacturers’ in-house standards.

The establishment of SP3-08 was deferred pending receipt of documentation in conformance with established WHO procedures. The Committee proposed that the endorsement of this project be conducted as a joint International Federation of Clinical Chemistry (IFCC) and WHO initiative to replace SP3-07.
5.2 Proposed new projects – blood products and related substances

5.2.1 Replacement of the Second WHO International Reference Preparation for serum IgE

To facilitate and improve transparency in the priority-setting process for new projects, a simple tool had been developed that described their salient features. The resulting documentation provided a means for the Committee and other stakeholders to review and comment on proposed new projects that were under consideration. Among the proposals made in document WHO/BS/11.2179 was the development of a replacement for the Second WHO International Reference Preparation for serum IgE.

Allergic disorders are common and their incidence is increasing. Serum IgE measurements are essential for the diagnosis and management of allergic disease. There is frequent demand for the current Reference Preparation which is used by assay manufacturers and clinical laboratories to standardize in vitro diagnostic tests for allergen-specific IgE in serum. As stocks of the Second WHO International Reference Preparation for serum IgE were nearing exhaustion, the preparation of a replacement consisting of pooled sera from patients with allergic disease was proposed, calibrated against the current Reference Preparation in an international collaborative study.

The Committee endorsed this proposal.

5.2.2 Replacement of the Third WHO International Standard for plasmin

Plasmin is the key enzyme responsible for the digestion of fibrin clots, and there is renewed interest in its use as a thrombolytic drug. The Third WHO International Standard for plasmin is currently used to estimate the direct relative potency of plasmin, and can also be applied to the wider standardization of fibrinolysis assays.

As stocks of the Third WHO International Standard for plasmin were now low, a replacement preparation was needed. The candidate replacement would consist of therapeutic-grade plasmin manufactured from human plasma. A truncated form of plasmin and full-length recombinant plasmin may also be included in the proposed collaborative study (WHO/BS/2011.2179) to provide valuable information on the utility of the replacement standard for their potency estimation. Value assignment of the replacement standard in IU would involve conventional chromogenic and fibrin clot-based assays relative to the current International Standard. However, the determination of molar concentration by active-site titration would also be investigated. The objective was to establish a replacement for the current International Standard in 2013.

The Committee endorsed this proposal.
5.2.3  **Haemoglobin A2**

The quantification of haemoglobin A2 (HbA2) is essential for the routine diagnosis of the beta thalassaemia trait, and hence for the identification of couples at risk of having a child with beta thalassaemia major. The First WHO International Reference Reagent for haemoglobin A2 (89/666) is currently the only international reference material available for the control of test procedures.

A number of issues exist in relation to the current situation. First, only one HbA2 level is covered by the reference material even though the calibration and control of routine methods would benefit from the use of at least two levels. More information on the methods used to establish the preparation should be made available. In addition, there exists a potential commutability issue, with an observed discrepancy reported between two high-performance liquid chromatography (HPLC) methods relating to a patient sample. There is also a lack of stability data on the current reference material. Finally, there may be a need to remind manufacturers that the current preparation is for the control of test procedures and not for calibration.

The IFCC had now established a working group on the standardization of HbA2 with the objective of defining an international reference system, including reference procedures and primary and secondary reference materials, in collaboration with the Institute of Reference Materials and Measurements (IRMM). It was suggested by the IFCC/IRMM that the First WHO International Reference Reagent for haemoglobin A2 (89/666) might be withdrawn when a new reference material had been developed with the IRMM.

The issue of commutability of the current International Reference Reagent was considered to be unresolved due to insufficient information. It was agreed that further information be obtained from diagnostics manufacturers on the use of this reagent, and on any related issues. It was also suggested that the information provided in the instructions for use for the current International Reference Reagent should be updated.

The Committee recommended that WHO communicate its procedures to the IFCC/IRMM for the establishment of international reference materials and standards. The Committee further advised that the instructions for use for the current International Reference Reagent be critically reviewed.

6.1 WHO International Standards and Reference Reagents – in vitro diagnostic device reagents

6.1.1 Third WHO International Standard for HIV-1 for NAT-based assays

Currently the Second WHO International Standard for HIV-1 for assays based on nucleic acid amplification techniques (NATs) is in place but stocks are running low due to high demand for this material. Therefore, a replacement standard (10/152) comprising heat-inactivated (1 hour at 60 °C) HIV-1 subtype B virus diluted in citrated human plasma had been prepared. Heat inactivation had been shown to have no negative effect on the detectability of HIV RNA by NAT-based assays. After lyophilization, the candidate standard was characterized in an international collaborative study (WHO/BS/2011.2178) together with the current International Standard, and the candidate represented in coded duplicates. Fifteen laboratories from 11 countries returned 17 data sets from 10 different assay systems (six quantitative and four qualitative). The results showed excellent correlation between the different assays used. Based on the results of this study, it was proposed that candidate material 10/152 was suitable as a replacement standard and should be established as the Third WHO International Standard for HIV1-RNA for NAT-based assays and be assigned a unitage of 185 000 IU/ml (5.27 log_{10} IU/ml).

The Committee recommended the adoption of candidate material 10/152 as the Third WHO International Standard for HIV1-RNA for NAT-based assays, with an assigned unitage of 185 000 IU/ml.

6.1.2 Third WHO International Standard for hepatitis B virus for NAT-based assays

This International Standard is used by in vitro diagnostic device manufacturers, blood transfusion centres, control authorities and clinical laboratories to calibrate secondary reference materials and in the validation of hepatitis B virus NAT-based assays. As stocks of the Second WHO International Standard for hepatitis B virus for NAT-based assays are diminishing a replacement is required.

Two candidate materials were therefore prepared from the same source (Eurohep R1 preparation; genotype A2) as that used for the current International Standard with a targeted concentration of 10^6 IU/ml. Both bulk materials were lyophilized as 0.5 ml aliquots, with post-fill testing including assessments of residual moisture and oxygen content by non-invasive near-infrared (NIR) spectroscopy. A collaborative study (WHO/BS/2011.2170) involving 16 laboratories from nine countries was conducted to evaluate the suitability and potency of
the two candidate freeze-dried preparations (NIBSC codes 10/264 and 10/266) in parallel with the Second WHO International Standard for hepatitis B virus for NAT-based assays using a range of such assays. When the results were expressed in IU/ml directly relative to the concurrently tested International Standard, the overall mean estimates from the quantitative assays were 5.93 (10/264) and 5.98 (10/266) log<sub>10</sub> IU/ml. The results of qualitative assays were characterized by higher levels of variation.

It was proposed that the candidate standard 10/264 be established as the Third WHO International Standard for hepatitis B virus DNA for NAT-based assays, with an assigned potency of 850 000 IU/ml (~5.93 log<sub>10</sub> IU/ml). It was noted that the second candidate (10/266) would also be a suitable potential replacement in due course, depending upon ongoing stability assessments.

The Committee recommended the adoption of preparation 10/264 as the Third WHO International Standard for hepatitis B virus DNA for NAT-based assays, with an assigned potency of 850 000 IU/ml.

6.1.3 First WHO International Reference Panel for hepatitis B virus genotype for HBsAg assays

Infection with the hepatitis B virus (HBV) is a major global health problem and the most common cause of liver cirrhosis and hepatocellular cancer. About two billion people worldwide have been infected with the virus and about 350 million live with chronic infection. An estimated 600 000 people die each year due to the chronic consequences of hepatitis B. The HBV is preferentially transmitted through contact with blood or other body fluid from an infected person. Sensitive screening and accurate diagnostic assays play a crucial role in the prevention and management of the disease.

The current International Standards for hepatitis B surface antigen (HBsAg) and for HBV DNA are both based on HBV-genotype A2 material. Concerns have been raised over the extent to which such preparations are representative of other HBV-genotypes (A–H) which are widely distributed globally, with clear regional variations observed in their distribution. In order to address this issue, two HBV-genotype panels were designed for use with HBV NAT-based assays or HBsAg assays. The first HBV-genotype panel, designed for use with HBV NAT-based assays, was established by the 2009 Committee. The HBV-genotype panel for use with HBsAg assays consists of 15 members representing the HBV genotypes A(3), B(2), C(3), D(3), E, F(2) and H. The panel members were derived from plasma samples collected worldwide with the majority of infectious HBV particles removed by ultracentrifugation. The HBsAg content of the source materials was determined by different methods, including chemiluminescent immunoassay, quantitative immune electrophoresis and antigen purification. The resulting HBsAg content was
expressed in IU/ml, PEI u/ml and ng/ml. Following dilution of the source materials, aliquoting and lyophilization, the panel members were characterized against the current First WHO International Standard for hepatitis B surface antigen. A resulting collaborative study (WHO/BS/2011.2180) resulted in 28 data sets from 15 laboratories, covering 20 different HBsAg assays. The results obtained demonstrated the consistent detection of HBV genotypes A–F and H by the majority of the test kits investigated, with some assays showing genotype-dependent effects on detection efficiency. This panel would be a very useful tool for regulatory authorities in assessing the relative HBsAg-detection efficiency in regard to HBV genotypes prevalent in their respective regions.

The Committee adopted the First WHO International Reference Panel for hepatitis B virus genotypes for use with HBsAg assays on the understanding that the instructions for use would be revised in compliance with the format used for the HBV genotype panel for use with HBV NAT-based assays.

6.1.4 Fourth WHO International Standard for hepatitis C virus for NAT-based assays

Infection with the hepatitis C virus (HCV) remains a major public health problem worldwide. Approximately 130–170 million people (2.2–3% of the world’s population) are infected with HCV. Chronic infection can lead to liver cirrhosis, liver failure and hepatocellular carcinoma. Although, the introduction of routine screening for HCV antibody from the late 1980s greatly reduced the risk of transmission via infected blood and blood products, there remained a major risk of transfusion-transmitted infection due to window-period donations. As a result, NAT-based assays were introduced to detect HCV RNA in human plasma as part of the safety testing of blood and blood products, and are now mandated in many parts of the world. HCV NAT-based assays are also widely used in the clinical management of HCV, particularly for diagnosing infection and monitoring response to antiviral therapies. A range of both commercial and laboratory-developed NAT-based assays are currently in use.

It is proposed that the current Third WHO International Standard for hepatitis C virus for NAT-based assays now be replaced by the freeze-dried preparation 06/102. This proposed replacement was prepared in 2006 from the same bulk as the current International Standard (06/100) but was filled and freeze-dried in a separate processing run. Both preparations containing hepatitis C virus (HCV) genotype 1a from anti-HCV-negative window phase donations were characterized in a worldwide collaborative study in 2007, and exhibited HCV-RNA concentrations of 5.19 (06/100) and 5.41 log_{10} IU/ml (06/102). In a more recent study (WHO/BS/2011.2173) seven laboratories from five countries participated in evaluating the potency and real-time stability of 06/102 by comparing it with both the current (06/100) and previous (96/798)
Reference Preparations. Surprisingly the mean potency estimates were 5.24, 5.08 and 5.05 log₁₀ IU/ml respectively – for the samples 06/102 and 06/100 there was a drop in potency of approximately 0.2 log₁₀ when compared to the results obtained in the original 2007 collaborative study, while sample 96/798 appeared to be still stable. Subsequent investigations provided evidence of the degradation of HCV RNA in 06/102 and 06/100 under elevated temperature during shipping, rather than a deterioration of the product during storage at –20 °C. Consequently, the candidate preparation is considered as a suitable replacement Fourth WHO International Standard for hepatitis C virus for NAT-based assays when maintained at or below –20 °C. Furthermore, it was agreed that the concentration determined in the 2007 collaborative study should be applied.

It was therefore proposed that the candidate sample 06/102 be established as the Fourth WHO International Standard for hepatitis C virus RNA for NAT-based assays with an assigned potency of 260 000 IU/ml (~5.41 log₁₀ IU/ml) when reconstituted in 0.5 ml of nuclease-free water. As shipment with dry ice and storage of the material at –20 °C would be a vital requirement, it was further suggested that priority should be given to its future replacement with a temperature-stable preparation. The problem of air entry into crimp-sealed vials during storage was also discussed, and it was agreed that efforts should be made to investigate the stability of other International Standards stored in such vials.

The Committee recommended the adoption of preparation 06/102 as the Fourth WHO International Standard for hepatitis C virus RNA for NAT-based assays, with an assigned potency of 260 000 IU/ml.

6.1.5 First WHO International Standard for hepatitis E virus for NAT-based assays

Hepatitis E virus (HEV) is a major cause of acute hepatitis and raises major public health concerns in endemic areas. HEV is also an emerging (or more-recognized) infection in developed countries. Certain patient populations (for example, individuals with liver disease, pregnant women and immune-suppressed patients) suffer from increased mortality rates associated with HEV infections. Chronic HEV infections are also being increasingly recognized. Viral load testing is important in the evaluation of antiviral therapy regimes, while NAT-based assays are also used as sensitive assays for the detection of recent infection. In humans four main HEV genotypes (1–4) have been described, with two (3 and 4) also associated with animal infections.

An initial collaborative study involving 20 laboratories from 10 countries was conducted to assess the consistency of results obtained by HEV NAT-based assays when testing dilution series of four HEV-positive human plasma materials. Wide variations in assay sensitivity (typically ranging from 100–1000-fold differences) were observed between different NAT-based assays, with significant
variations in reporting of results. A candidate International Standard preparation (6329/10) was selected from this study and alongside another preparation designed as national standard for Japan (HEV genotype 3b) was chosen for detailed characterization in a second collaborative study involving 24 laboratories from 10 countries (WHO/BS/2011.2175). This study confirmed the feasibility of standardizing HEV NAT-based assays using the candidate preparation 6329/10. Commutability would be addressed in a further study where the candidate would be tested in selected assays alongside a set of HEV-positive patient samples. The current report would also be supplemented with a consideration of the stability of the preparation (preliminary data are already present), more detailed lyophilization conditions and a summary of the potency data in tables. It was proposed that 6329/10 be established as the First WHO International Standard for hepatitis E virus RNA for NAT-based assays with an assigned unitage of 250 000 IU/ml.

The Committee recommended the adoption of preparation 6329/10 as the First WHO International Standard for hepatitis E virus for NAT-based assays, with an assigned unitage of 250 000 IU/ml and with the proviso that the report would be updated as described.

6.1.6 First WHO International Reference Standard for anti-Trypanosoma cruzi antibodies

Chagas disease (human American trypanosomiasis) is caused by infection with the protozoan parasite *Trypanosoma cruzi* which is endemic in Latin America and is transmitted by triatomine bugs. It is estimated that around 10 million people are currently infected by *T. cruzi*, and more than 10 000 people die from its chronic clinical manifestations every year. Large-scale insect-control programmes in Central and South American countries, together with the mandatory screening of blood donations for anti-*T. cruzi* antibodies, have reduced the incidence and prevalence of the disease. Two groups of *T. cruzi* are differentiated, with *T. cruzi I* being mainly prevalent north of the Amazon basin (for example in Mexico) whereas *T. cruzi II* is more prevalent in southern Latin American countries (for example, Brazil and Chile). However, these two groups have still not been characterized for genetic and antigenic differences. A small number of anti-Chagas seropositive plasma samples obtained respectively from autochthonous blood donors from either Mexico or from Brazil and/or Chile were pooled to result in presumably anti-*T. cruzi I*-positive and anti-*T. cruzi II*-positive preparations respectively. The two preparations were aliquoted, lyophilized and provided as coded materials for a global collaborative study involving 24 laboratories from 16 countries (WHO/BS/2011.2181) with the inclusion of different serological assays used for anti-Chagas screening, serodiagnosis or supplemental information provision. The preparations were found suitable for assessing the analytical sensitivities of the assays.
It was proposed to assign distinct unitages of 1 IU/ml (undiluted, after reconstitution) to each of the materials and to establish them as the First WHO International Reference Standard for anti-*Trypanosoma cruzi* antibodies to be used concomitantly. It was understood that neither the anti-*T. cruzi* II unitage of the anti-*T. cruzi* I reference standard nor the anti-*T. cruzi* I unitage of the anti-*T. cruzi* II reference standard had been determined.

Preparation 09/186 is defined as the anti-*T. cruzi* antibody standard representative of the region where *T. cruzi* II is predominant, while preparation 09/188 is defined as the anti-*T. cruzi* antibody standard representative of the region where *T. cruzi* I is predominant.

The Committee recommended the concomitant adoption of preparations 09/186 and 09/188 as the First WHO International Reference Standard for anti-*Trypanosoma cruzi* antibodies, each with an assigned unitage of 1 IU/ml and subject to final editing of the instructions for use.

6.1.7 First WHO International Standard for Epstein–Barr virus for NAT-based assays

Both proficiency-testing programmes and feedback from the clinical diagnostic community have indicated a need for the standardization of Epstein–Barr virus (EBV) viral load assays. Viral load measurements are important in the prevention, diagnosis and monitoring of EBV infections. With non-standardized NAT-based assays currently in use it is not easily possible to compare clinical practice at different sites and to standardize patient management. Whole B95-8 virus was chosen as a candidate strain in order to allow for the standardization of the entire assay, including extraction. The candidate preparation (09/260; ~1×10⁷ copies/ml) was formulated in universal buffer for subsequent dilution in the appropriate sample matrices used in the routine testing of plasma, serum and whole blood. The candidate was evaluated both as lyophilized and frozen liquid preparations alongside EBV-positive Raji and Namalwa cells.

A collaborative study was conducted involving 28 laboratories from 16 countries (WHO/BS/2011.2172) selected for experience in EBV NAT-based assays and for geographical representativeness, and including in vitro diagnostic device manufacturers and clinical, reference and research laboratories. In total, 38 datasets (36 quantitative assays and two qualitative assays) were returned and used for the statistical evaluation. The analysis confirmed the wide range of results reported for the same preparation by different assays (as copies, genome equivalents etc.) independent of the nature of the specimen (virus or cells). When lyophilized B95-8 EBV preparation was used as a common standard material, the results for the frozen liquid preparation became quite consistent between the different assays. However, for the cell preparations (Raji and Namalwa) this standardization effect appeared to be less pronounced despite a clear
improvement in the overall consistency of result reporting for the different assays. This clear difference in the consistency of reporting plasma or cell-associated loads of EBV by different NAT-based assays will be investigated further by NIBSC. At a Standardization of Genome Amplification Techniques (SoGAT) Clinical Diagnostics meeting, potential users proposed the establishment of this preparation as the WHO standard despite the standardization limitations observed in the analysis of cell-associated EBV. It was therefore proposed that the candidate standard 09/260 be established as the First WHO International Standard for Epstein–Barr virus for NAT-based assays with an assigned unitage of $5 \times 10^6$ IU when reconstituted in 1 ml of nuclease-free water. The potential need to develop an EBV type-2 standard was noted.

It was also suggested that the final edited instructions for use should address: (a) the limited ability of the standard to control for efficiency of extraction of cell-associated EBV DNA; (b) the possibility that methylation of cell-associated virus DNA could artefactually reduced detection, compared with NAT-based assays; and (c) the fact that the current standard is a type-1 EBV.

The Committee recommended the adoption of preparation 09/260 as the First WHO International Standard for Epstein–Barr virus for NAT-based assays, with an assigned unitage of $5 \times 10^6$ IU when reconstituted in 1 ml of nuclease-free water.

6.2 Proposed new projects – in vitro diagnostic device reagents

6.2.1 Third Meeting of WHO Collaborating Centres for Biological Standards

The Third Meeting of WHO Collaborating Centres for Biological Standards was hosted by NIBSC on 7–8 March 2011 and was attended by representatives from FDA, NIBSC, PEI and WHO. The aim of the meeting was to strengthen cooperation between the collaborating centres, and to share knowledge and resources in order to advance the field of standardization of in vitro diagnostic devices.

The commutability of Reference Preparations for use in the calibration of diagnostic tests was reported to be an important area of scientific debate. The collaborating centres proposed that WHO convene a meeting to review the principles of commutability and their applicability to WHO International Standards. Meeting participants would ideally include experts in the field of metrology, with the overall goal of the meeting being the development of guidelines on evaluating commutability during the validation of international reference materials for in vitro diagnostic devices (including clinical diagnostics and infectious-disease tests). Updates were then provided of ongoing collaborative studies to evaluate new or replacement standards.

The Committee endorsed the proposal to convene a meeting on the commutability of Reference Preparations for in vitro diagnostic devices.
6.2.2 Replacement of the First WHO International Standard for hepatitis A virus for NAT-based assays

The hepatitis A virus (HAV) is responsible for approximately half of all cases of hepatitis diagnosed worldwide. It is also recognized as one of the most important human foodborne pathogens.

The current International Standard (00/560) was prepared from a wild-type isolate (representing HAV genotype 1a) derived from human plasma, and was filled and freeze-dried in 2001. A potential replacement candidate (00/562) was also prepared from the same bulk. At the current rate of depletion, it is estimated that the stock of 00/560 will need to be replaced in ~2–3 years. However, following its storage at +4 °C for five years and 10 months, a stability assessment of samples of 00/562 suggested that there was some loss of potency (~1 log_{10}). Another more recent stability assessment of accelerated thermal degradation of samples of 00/560 and 00/562 stored at +4 °C for nine years and 10 months also suggested that 00/562 might not be the optimal replacement candidate.

NIBSC was therefore in the process of sourcing HAV RNA-positive (genotype 1a) human plasma sample(s) negative for other viral markers (such as HBV, HCV, HIV and B19V) for the design and manufacturing of a suitable replacement standard. Once a suitable material had been identified, a collaborative study involving approximately 20 laboratories would be conducted.

The Committee endorsed the proposed project (WHO/BS/2011.2179) to develop a replacement for the First WHO International Standard for hepatitis A virus for NAT-based assays.

6.2.3 Replacement WHO International Standard for hepatitis B e-antigen

HBV infection is a major health problem worldwide with an estimated 350 million chronic carriers. It is estimated that around one third of the human population has encountered the virus. Hepatitis B e-antigen (HBeAg) is a diagnostic marker for HBV infectivity that circulates in the blood when the virus is actively replicating, and is thus indicative of both viral replication and potential infectivity. The marker is used to determine the status of HBV infection and to monitor patients. European legislation (Common Technical Specifications 2009/886/EC) requires traceability of in vitro diagnostic test results to a higher-order standard. Since 1982 the PEI has provided an HBeAg standard for the determination of the analytical sensitivity of HBeAg tests used by manufacturers worldwide. There is a continuous need for the standard, with the material historically having been assigned a PEI unit (u/ml). The PEI material now had to be replaced, ideally by an International Standard expressed in IU/ml.

The Committee endorsed the proposed project (WHO/BS/2011.2179) to develop a replacement WHO International Standard for HBeAg.
6.2.4 Replacement WHO International Standard for hepatitis B e-antibodies

HBV infection is spread worldwide with around two billion people having been infected with the virus and about 350 million people living with chronic infection. The diagnosis of HBV requires a combination of various tests including the detection of antibodies to hepatitis B e-antigen (anti-HBeAg). The anti-HBeAg test is particularly meaningful in association with the HBeAg test for monitoring the course of HBV infection.

Anti-HBeAg preparations are used for the validation, standardization, quality control and comparison of anti-HBe tests. The PEI anti-HBeAg reference serum established in 1982 is used by in vitro diagnostic device manufacturers worldwide. As with HBeAg there is a continuous need for an anti-HBeAg standard with the material historically having been assigned a PEI unit (u/ml). The PEI material now had to be replaced, ideally by an International Standard expressed in IU/ml.

The Committee endorsed the proposed project (WHO/BS/2011.2179) to develop a replacement WHO International Standard for anti-HBeAg.

6.2.5 First WHO International Reference Panel for hepatitis E virus genotypes for NAT-based assays

Hepatitis E virus (HEV) is represented by a single serotype but can be classified into at least four main genotypes (1–4). While HEV genotypes 1 and 2 are exclusively detected in humans, genotypes 3 and 4 are found in both humans and in a range of other animals (for example, pigs, wild boar, deer and rodents). The geographical distribution of HEV genotypes is complex and there is approximately 74% nucleotide identity between genotypes. Once the First WHO International Standard for hepatitis E virus for NAT-based assays has been established, a bias towards the sensitive detection of HEV genotype 3a (as included in the WHO candidate preparation) cannot be ruled out.

A tool to assess genotype specificity and the relative genotype sensitivity of HEV NAT-based assays is therefore needed, especially for assays used in regions with a high prevalence and incidence of one or more genotypes other than genotype 3a. Laboratories in such regions should be included in the study.

The HEV genotype panel project aims to incorporate different HEV genotypes and sub-genotypes into a reference panel which may be very helpful for assessing assay features. Currently, different materials representing three genotypes are already available at PEI, and efforts are being undertaken to obtain source material representing the missing genotype 2.

The Committee endorsed the proposed project (WHO/BS/2011.2179) to develop a First WHO International Reference Panel for hepatitis E virus genotypes for NAT-based assays.
6.2.6 **Replacement of the First WHO International Reference Panel for HIV-1 subtypes**

The First WHO International Reference Panel for HIV-1 subtypes (01/466) was established by the Committee in 2003. This original panel was a liquid preparation stored at –80 °C and was composed of different subtypes of HIV-1 (A, B, C, D, AE, F, G and AGH) as well as representatives of group N and O. The replacement panel would be of the same composition and from the same isolates but would undergo heat inactivation (1 hour at 60 °C) and lyophilization. Full-length sequences had been published previously for these isolates and the sequences would be reconfirmed by NIBSC. A full collaborative study would also be performed.

The Committee endorsed the proposed project (WHO/BS/2011.2179) to develop a replacement for the First WHO International Reference Panel for HIV-1 subtypes.

6.2.7 **First WHO International Reference Panel for HIV-1 circulating recombinant forms**

The diversity of HIV was well documented with increasing epidemiological evidence of inter-subtype recombinant forms of HIV-1 strains circulating in different parts of the world. It was now widely appreciated that the recombinant nature of HIV had allowed for the evolution of circulating recombinant forms (CRFs) of HIV in which the virus may exhibit different genotypes across genomic regions. There was therefore a need to establish respective panels to be able to perform state-of-the-art assessments of HIV-1 using NAT-based assays. It was intended that high-titre stocks (grown in cell culture) of HIV-1 subtypes G, H, J, K, group O and a range of CRFs would be used as panel members. These would be diluted in negative human plasma and heat inactivated prior to lyophilization. The panel would be evaluated in a full international collaboration involving countries known to have a variety of genotypes in circulation.

The Committee endorsed the proposed project (WHO/BS/2011.2179) to develop a First WHO International Reference Panel for HIV-1 circulating recombinant forms.

6.3 **Ongoing stability monitoring**

6.3.1 **Prostate-specific antigen**

The principle application of International Standards in this area is the calibration of diagnostic assays for prostate-specific antigen (PSA). As part of their original establishment, PSA standards were recommended for ongoing stability monitoring. In the absence of frozen baselines for such standards, a second accelerated degradation study was performed (WHO/BS/2011.2161). At 12 years
post-fill, ampouled materials (stored at elevated temperatures for 13 months) were compared with ampoules stored at –20 °C. A lack of degradation at +4 °C and +20 °C precluded a prediction of degradation rate using the Arrhenius equation but supported the predictions on stability made at establishment. For samples stored at –20 °C the predicted degradation rates were 0.042% per year (PSA free) and 0.027% per year (PSA 90:10).

As the remaining stocks of these standards were low and were predicted to last no more than 4–5 years, no further stability assessment was proposed.

The Committee noted that these stability studies had confirmed the predictions made at the time of standard establishment. The Committee asked that this information be included in shipments of this material.
7. International reference materials –
biotherapeutics other than blood products

All reference materials established at the meeting are listed in Annex 8.

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

7.1.1 First WHO International Standard for transforming growth factor beta-3

Transforming growth factor beta-3 (TGF-β3) is a member of the TGF-β superfamily (30 proteins) which includes activins, bone morphogenetic proteins and growth and differentiation factors. Three closely related isoforms exist in mammals – TGF-β1, -β2 and -β3 – each is derived from a distinct gene but exhibits greater than 70% sequence homology and similar functional responses in vitro. Produced by several immune and non-immune cell types such as fibroblasts, endothelium, smooth muscle cells, TGF-β acts in an autocrine and paracrine manner to regulate multiple cellular processes, such as immune function, proliferation, differentiation, extracellular matrix production, migration and survival.

The therapeutic potential of TGF-β3 in the treatment of oral mucositis and in the prevention and reduction of scarring following surgical procedures and wound repair has been shown in various phase I/II clinical trials. Additionally, TGF-β3 appears to be a potentially useful growth factor in engineered organogenesis and in the context of regenerative medicine.

Based on its activities (pro-inflammatory, immunosuppressive), TGF-β has also been implicated in many human diseases, including certain cancers, onset of several autoimmune diseases including joint destruction in arthritis and in pathogenesis of fibrotic diseases associated with skin, lung, liver and eye (cornea).

A reference standard for TGF-β3 could facilitate measurement of the potency and stability of therapeutic preparations or for preparations in use in regenerative medicine, its antagonists and in addition, measurement of TGF-β3 levels for research purposes. Currently, a Reference Reagent for TGF-β3 (98/608) is available from NIBSC with an arbitrary unitage of 10 000 IU/ampoule. The objective of the current study (WHO/BS/2011.2163) was therefore to characterize a candidate International Standard for the bioassay of human TGF-β3 and assign a unitage for its activity.

The results clearly indicated that the candidate preparation 09/234 was suitable for use as an International Standard for TGF-β3. It was proposed that the new international unit should preserve continuity with the existing NIBSC Reference Reagent (98/608) with a proposed value of 19 000 IU/ampoule to be assigned.
The Committee endorsed the proposal to establish the preparation 09/234 as the First WHO International Standard for transforming growth factor beta-3, with an assigned unitage of 19 000 IU/ampoule. The Committee requested that future studies be conducted with mammalian cell expressed material, when it became available, to assess the performance of the standard.

7.2 Proposed new projects – biotherapeutics other than blood products

7.2.1 Replacement of the First WHO International Standard for interleukin-2

The current First WHO International Standard for interleukin-2 (86/504; 100 IU/ampoule) is used for the potency labelling of interleukin-2 products approved for the treatment of metastatic renal cell carcinoma and metastatic melanoma patients. Additionally, based on its ability to induce proliferation of CD4+ cells, it has been clinically tested in HIV-positive patients either alone or as combination therapy with antiviral agents. Based on promising results from preliminary studies using combination therapy, further clinical trials were ongoing in HIV-positive patients. As stocks of the standard preparation would be almost exhausted in 2014 a replacement was required.

A proposed multi-centre international study (WHO/BS/2011.2177) would include the evaluation of two candidate preparations from the previous collaborative study, predominantly using bioassays. Unitage would be assigned relative to the current International Standard. One of the candidate rDNA-derived human interleukin-2 (86/564) preparations from the previous collaborative study was currently being distributed with no reported issues. Limited stability studies after 22 years of lyophilization had indicated no loss of biological activity.

The Committee endorsed the proposal to evaluate the suitability of a new preparation of interleukin-2 as the Second WHO International Standard for interleukin-2.

7.2.2 Replacement of the Second WHO International Standard for recombinant human erythropoietin

Erythropoietin (EPO) is used globally in the treatment of anaemia. Defining a standard IU value for EPO activity enables manufacturers to correctly label the potency of therapeutic EPO products used to treat anaemia in end-stage renal disease and anaemia associated with cancer and other diseases. As stocks of the current Second WHO International Standard for recombinant human erythropoietin (rhuEPO) were running low there was an urgent requirement for a new International Standard.

Participants in the proposed collaborative study (WHO/BS/2011.2177) would be asked to calibrate the candidate standard in terms of the current standard.
using polycythaemic or normocythaemic mouse bioassay. Confirmatory data would also be requested from physicochemical analyses.

The Committee endorsed the proposal to evaluate the suitability of a new preparation of rhuEPO as the Third WHO International Standard for recombinant human erythropoietin.

### 7.2.3 Replacement of the Fourth WHO International Standard for urinary follicle-stimulating hormone and urinary luteinizing hormone

Urinary follicle-stimulating hormone (FSH) and luteinizing hormone (LH) products are manufactured and marketed worldwide. The current International Standard defines the IU value which is essential for the correct potency labelling of the therapeutic preparations of urinary FSH/LH used to treat anovulation in women and hypo- or normo-gonadotrophic hypogonadism in men. It is also used for ovarian hyperstimulation as part of assisted reproductive technologies. Strong demand for low-cost preparations, particularly for assisted reproductive technologies in countries where it is not funded by government or healthcare plans, necessitates effective standardization.

As stocks of the current Fourth WHO International Standard for urinary follicle-stimulating hormone and urinary luteinizing hormone (98/704) were almost exhausted there was an urgent requirement for a new International Standard.

Participants in the proposed collaborative study (WHO/BS/2011.2177) would be asked to calibrate the standard in terms of the current standard using rat ovarian weight gain (for FSH) and immature rat seminal vesicle weight gain (for LH) bioassays. Confirmatory data would also be requested from in vitro bioassays.

The Committee endorsed the proposal to evaluate the suitability of a new preparation of FSH/LH as the Fifth WHO International Standard for urinary follicle-stimulating hormone and urinary luteinizing hormone.

### 7.2.4 Replacement of the Second WHO International Standard for luteinizing hormone (human, pituitary)

Licensed LH immunoassays are an established component of the battery of clinical endocrinology tests used in the diagnosis of a range of diseases. For example, measurements of LH are used in the diagnosis of hypothalamic, pituitary or gonadal dysfunction. In addition, LH levels are used to determine menopause, pinpoint ovulation and monitor endocrine therapy. As stocks of the current Second WHO International Standard for luteinizing hormone (human, pituitary) are running low there is a requirement for a replacement International Standard for the calibration of LH immunoassays.
As part of the proposed collaborative study (WHO/BS/2011.2177) the candidate preparation would be value-assigned against the current standard using commercial immunoassay methods.

The Committee endorsed the proposal to evaluate the suitability of a new preparation of LH as the Third WHO International Standard for luteinizing hormone, human, pituitary, and noted that this would be the last natural LH standard.
8. International reference materials – antibiotics

All reference materials established at the meeting are listed in Annex 8.

8.1 WHO International Standards and Reference Reagents – antibiotics

8.1.1 Third WHO International Standard for dihydrostreptomycin

Dihydrostreptomycin is an antibiotic consisting of a hydrogenated form of streptomycin. It is used in the prophylaxis of tuberculosis and tularaemia, and for the treatment of infections caused by Gram-negative bacteria. By preventing the initiation of protein synthesis through binding to the 30S subunit of the bacterial ribosome it results in the death of microbial cells. Humans and other mammals have structurally different ribosomes from bacteria thus explaining the selective action of this antibiotic.

The Second WHO International Standard for dihydrostreptomycin was established in 1964 on the basis of an international collaborative study. A potency of 820 IU/mg was assigned, with each ampoule containing approximately 200 mg. As stocks of the current standard are becoming exhausted, the EDQM – which acts as the WHO custodian laboratory for antibiotics – was requested by the Committee to undertake the appropriate steps for its replacement.

Using the current standard as a reference, participating laboratories in an international collaborative study (WHO/BS/2011.2176) were requested to estimate the potency and suitability of a candidate standard (EDQM code number ISA_42688). It was concluded that the candidate material should be proposed as the Third WHO International Standard for dihydrostreptomycin and assigned an activity value of 19 425 IU per vial.

The Committee recommended that ISA_42688 be established as the Third WHO International Standard for dihydrostreptomycin, with an assigned activity value of 19 425 IU/vial.

8.2 Proposed new projects – antibiotics

8.2.1 Replacement of the First WHO International Standard for neomycin B

Neomycin B is a globally marketed antibiotic which requires the use of an International Standard to enable quality-control assessments to be undertaken by manufacturers and NCLs. It was envisaged that stocks of the First WHO International Standard for neomycin B would be exhausted by 2012–2013.

Manufacturers and public control laboratories from all over the world would be invited to participate in a collaborative study (WHO/BS/2011.2177) to establish a replacement standard.

The Committee endorsed the proposal to evaluate the suitability of a candidate Second WHO International Standard for neomycin B.
8.2.2 Replacement of the Second WHO International Standard for neomycin

Neomycin is another globally marketed antibiotic which requires the use of an International Standard to enable quality-control assessments to be undertaken by manufacturers and NCLs. It was envisaged that stocks of the Second WHO International Standard for neomycin would be exhausted by 2012–2013.

Manufacturers and public control laboratories from all over the world would be invited to participate in a collaborative study (WHO/BS/2011.2177) to establish a replacement standard.

The Committee endorsed the proposal to evaluate the suitability of a candidate Third WHO International Standard for neomycin.
Annex 1

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

The Recommendations (previously called Requirements) and Guidelines published by WHO are scientific and advisory in nature but may be adopted by an NRA 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 WHO Technical Report Series as listed below. A historical list of Requirements and other sets of Recommendations is available on request from the World Health Organization, 20 avenue Appia, 1211 Geneva 27, Switzerland.

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

WHO Press
World Health Organization
20 avenue Appia
1211 Geneva 27
Switzerland
Telephone: +41 22 791 3246
Fax: +41 22 791 4857
E-mail: bookorders@who.int
Web site: http://www.who.int/bookorders

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

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

Abbreviated in the following pages to "TRS".
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# Annex 2

**Guidelines on the quality, safety and efficacy of dengue tetravalent vaccines (live, attenuated)**

Replacement of Annex 1 of WHO Technical Report Series, No. 932

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Guidelines published by WHO are intended to be scientific and advisory in nature. It is recommended that modifications 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 Guidelines set out below. To facilitate the international distribution of vaccine made in accordance with these Guidelines, a summary protocol for the recording of results of the tests is given in Appendix 1.
Introduction

These Guidelines are intended to provide national regulatory authorities (NRAs) and vaccine manufacturers with guidance on the quality, safety and efficacy of live tetravalent dengue vaccines currently under clinical development to facilitate their international licensure and use.
These Guidelines update the Guidelines for the production and quality control of candidate tetravalent dengue virus vaccines (live) (1). They should be read in conjunction with other WHO guidelines that are referred to in each part. The Guidelines cover dengue tetravalent vaccines (live, attenuated). Other types of dengue virus vaccines under development (e.g. subunit or inactivated vaccines) are outside the scope of these Guidelines. However, some guiding principles provided in these Guidelines (e.g. Part C on clinical evaluation) may be useful for the evaluation of other types of dengue vaccine. For quality control, guiding principles applicable to other types of vaccines – such as inactivated or subunit vaccines – are available elsewhere if the product in development shares similar manufacturing processes. For example, guidelines for human papillomavirus and hepatitis B vaccines may also be useful for subunit vaccines for dengue.

These Guidelines are based on experience gained from candidate dengue tetravalent vaccines (live, attenuated) that have been developed as described below, and will need to be updated as new data become available from additional studies. Part A sets out guidelines for manufacture and quality control. Guidelines specific to the nonclinical and clinical evaluation and environmental risk assessment are provided in parts B, C and D, respectively. Part E provides guidelines for NRAs. In the following section, brief overviews of dengue disease and dengue vaccine development at the time of preparing this document are provided as a scientific basis for each part.

**General considerations**

**Dengue viruses**

Dengue is a mosquito-borne disease and represents a major public health problem throughout the tropical world. The causative dengue viruses (DENVs) are members of the genus *Flavivirus*, within the family *Flaviviridae*. There are four serotypes (termed DENV-1 to DENV-4) and at least three genetic groups (genotypes) within each serotype.

All flaviviruses are lipid-enveloped, positive-sense, single-stranded RNA viruses, approximately 55 nm in diameter. The genome is capped at the 5’ terminus but does not have a poly A tract at the 3’ terminus, and is approximately 11 000 nucleotides in length. The virion RNA encodes a single open reading frame that is flanked by a 5’ untranslated region (UTR) and a 3’ UTR. The open reading frame is translated into a polyprotein that is co- and post-translationally cleaved to yield at least 10 proteins. Three structural proteins are derived by cleavages of the amino-terminal one third of the polyprotein: the capsid or core protein forms a “nucleocapsid” complex with virion RNA that lies within the lipid envelope. The premembrane (prM) and envelope (E) proteins are embedded in the lipid envelope via carboxy-terminal transmembrane domains.
and are displayed on the surface of virions. Cleavage of the carboxy-terminal two thirds of the polyprotein yields seven non-structural (NS) proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. NS3 encodes a serine protease in the N-terminal 180 amino acids and helicase, nucleotide triphosphatase, and RNA 5’-triphosphatase activities in the C-terminal region. NS5 encodes two functions: the first one third encodes a methyltransferase that sequentially methylates the N7 and 2’-O positions of the viral RNA cap using S-adenosyl-l-methionine as a methyl donor, and the remainder a RNA-dependent RNA polymerase. NS1 plays various roles in the virus replication cycle while NS2A, NS2B, NS4A and NS4B are all small hydrophobic proteins with the central region of NS2B required for the functioning of the NS3 protease.

Host range and transmission

DENVs are most commonly transmitted to humans by the bite of infected *Aedes aegypti* mosquitoes, which are highly domesticated and the primary mosquito vector. However, *Aedes albopictus* can also sustain human-to-human transmission. The drastic increase in the incidence of DENV infection in the Americas during the past 30 years is primarily due to the geographical spread of *Ae. aegypti* following the decline in vector-control efforts. The DENV that infects and causes disease in humans is maintained in a human-to-mosquito-to-human cycle and does not require a sylvatic cycle in nonhuman primates. Certain strains of DENV are known to be transmitted to nonhuman primates in western Africa and Malaysia. However, transmission to humans via mosquitoes from nonhuman primates is believed to be very limited.

Clinical and pathological manifestation in humans

Following infection resulting from the bite of an infected mosquito, the virus is thought to replicate in local dendritic cells. Subsequent infection of macrophages and lymphocytes is followed by entry into the bloodstream. Haematogenous spread is the likely mechanism for seeding of peripheral organs and the occasional reports of central nervous system infections, which can lead to symptomatic illness.

Most DENV infections are either asymptomatic or only mildly symptomatic. The incubation period of dengue can range from 3 to 14 days, but is generally 4–7 days. Most symptomatic DENV infections present with a sudden onset of fever accompanied by headache, pain behind the eyes, generalized myalgia and arthralgia, flushing of the face, anorexia, abdominal pain and nausea. Rash is common in dengue and can be macular, maculopapular, morbilliform, scarlatiniform or petechial in character. Rash is most often seen on the trunk, on the insides of the arms and thighs, and on plantar and palmar surfaces. Laboratory abnormalities that can be observed in dengue infection include leukopenia and thrombocytopenia.
Dengue illness is classified as (i) dengue with or without warning signs and (ii) severe dengue. A presumptive diagnosis of dengue can be made in a patient living in or travelling from a dengue-endemic area who has fever and at least two of the following clinical signs or symptoms: anorexia and nausea, rash, body aches and pains, warning signs, leukopenia, and a positive tourniquet test. “Warning signs” include abdominal pain or tenderness, persistent vomiting, clinical fluid accumulation, mucosal bleeding, lethargy or restlessness, liver enlargement of > 2 cm, or an increase in haematocrit concurrent with a rapid decrease in platelet count. Dengue illness should be classified as severe in a patient with presumptive dengue and any of the following: severe plasma leakage leading to shock or respiratory compromise, clinically significant bleeding, or evidence of severe organ involvement. Detailed case classification of dengue is provided in a separate WHO document (or its subsequent update) (2).

Nonhuman primate dengue virus infection

Natural hosts for DENV infection are humans and mosquitoes. Serological evidence from nonhuman primate studies indicates the existence of a sylvatic DENV cycle involving several species of mosquitoes and several monkey species. Although monkeys develop a viraemia and a neutralizing antibody response to DENV infection, they do not develop the haematological abnormalities seen in humans. However, in nonhuman primates (and AG129 mouse, see item below) primary DENV infections cause a leukopenia. Thrombocytopenia has been observed after a secondary infection (3–6).

In the rhesus macaque, viraemia typically begins 2–6 days after infection and lasts for 3–6 days (3, 5, 7). Virus spreads to regional lymph nodes and can be isolated from the skin, distant lymph nodes, and rarely from spleen, thymus and other body organs. The nonhuman primate model for DENV is useful for measuring the protection from viraemia conferred by vaccination or passively acquired antibody. Disadvantages of the nonhuman primate model include the lack of overt clinical signs of disease (4–6, 8).

Mouse dengue virus infection

Clinical isolates of DENV do not replicate well in genetically normal mice. However, mouse-brain-adapted DENVs can induce fatal encephalitis after intracranial inoculation of suckling mice. It has been demonstrated that adaptation of a DENV-2 isolate to neurovirulence in suckling mice correlated positively with attenuation of virulence in humans (9). Because of this ambiguity, the suckling mouse/encephalitis model is probably not useful for studying the safety or efficacy of candidate dengue vaccines. Nevertheless, it could be used to assess lot consistency (see sections A.3.2.5.5.2 and A.4.2.4.7). In recent years, both chimeric mice that are transplanted with human cells and severely immunocompromised
strains of mice have been used to elucidate the immune response to dengue infection and to study pathogenesis (4, 10, 11). Interferon receptor-deficient AG129 mice support replication of selected DENV strains which infect relevant cell and tissue types comparable to human infection (10, 12). AG129 mice have been used to investigate antibody-mediated protection. A strain of DENV-2 that has been adapted to AG129 mice by serial passage between mice and mosquito cells has a viscerotropic phenotype, causing thrombocytopenia and vascular leakage in the infected animals. The phenomenon of antibody-dependent enhancement of virus infection was observed in AG129 mice following passive transfer of anti-DENV-1 antibodies and challenge with the adapted strain of DENV-2 (10, 12, 13). The relevance of such an immunocompromised mouse model may, however, be limited with regard to vaccine evaluation (see section B.4.3).

Mosquito dengue virus infection

Vector competence refers to the efficiency with which the vector transfers infection between hosts. Typically, this is a product of vector susceptibility to infection, replication efficiency of the pathogen in the vector, and the sensitivity of the host to infection transmitted by vector contact. *Ae. aegypti* mosquitoes exhibit global variation in vector competence for flaviviruses. For example, in sub-Saharan Africa, a black “sylvan” subspecies (*Ae. formosus*) predominates. This mosquito has a low vector competence for flaviviruses due primarily to a midgut infection barrier (14). Once ingested in an infectious blood meal, DENVs should replicate in the midgut and disseminate to the salivary glands to facilitate transmission to a new host during feeding. In this process, the virus should overcome any midgut barrier that would limit replication and prevent spread of the virus to other tissues in the mosquito (6, 15, 16).

None of the live dengue vaccine preparations currently in the clinical trial phase of development is effectively transmitted by mosquito vectors (16, 17), because vaccine viruses replicate poorly in mosquito midgut epithelium and/or do not disseminate efficiently to the salivary glands, thereby effectively precluding transmission to humans (6, 18, 19). In addition, the low peak titre and very short duration of viraemia induced by these candidates in humans has been shown to render vaccinees relatively non-infectious for feeding mosquitoes. The net effect of these two phenomena is a drastic reduction in vector competence.

Populations at risk and global health importance

Dengue is the most rapidly spreading mosquito-borne viral disease in the world. Since 1955, the incidence of dengue and severe dengue reported to WHO has increased approximately 30-fold with increasing geographical expansion to new countries and from urban to rural settings. Approximately 3.5 billion people live in dengue-endemic countries which are located in the tropical and subtropical
regions of the world. An estimated 50 million dengue infections occur annually and the number of cases reported annually to WHO ranged from 0.4 million to 1.3 million in the decade 1996–2005 (2).

**Justification for vaccine development**

Prevention of dengue by vector control has proven to be very difficult and costly. While vector-control efforts should be sustained, vaccination holds substantial potential in the control of the disease. Hence, there is an urgent need to develop dengue vaccines, especially to protect people from disease in endemic countries.

**Development of candidate dengue vaccines**

Efforts to develop a vaccine against dengue have focused primarily on live, attenuated viruses, derived by serial passage of virulent viruses in tissue culture or via recombinant DNA technology that permits site-directed mutagenesis of the genome of a virulent parent strain or chimerization between flaviviruses. Success in the development, licensure and clinical use of live, attenuated flavivirus vaccines, such as yellow fever 17D vaccines and Japanese encephalitis SA14-14-2, suggests that a suitably attenuated live dengue vaccine could be highly efficacious. Other vaccine candidates, based on inactivated whole virus, subunits that include E protein, virus-like particles composed of prM and E proteins, and DNA vaccines that induce expression of DENV prM/E proteins, are in the nonclinical or early clinical stages of development.

There are no animal models that completely mimic the protean manifestations of dengue. The lack of a suitable animal model makes it difficult to assess the efficacy of vaccine candidates and to identify or establish possible correlates of protection in vivo. Therefore, the protective capacity of any vaccine candidate will be finally defined by its ability to protect humans from dengue febrile illness (DFI). Results of nonclinical studies using monkeys and susceptible mouse strains suggest, however, that protection from dengue is best correlated with the presence of virus-neutralizing antibodies (3, 20–26). Studies in which vaccinated volunteers were challenged with dengue viruses have been conducted in the past but are not a required part of currently recommended clinical development programmes.

There is general agreement that DENV vaccines should ideally induce protective neutralizing antibodies to each of the four serotypes simultaneously. In theory, a tetravalent immune response would protect against all DFI and would also reduce or eliminate the risk of a phenomenon termed antibody-dependent enhancement of disease, which is thought to be one of the mechanisms that predispose to severe forms of dengue.

Several strategies have been employed to derive candidate live, attenuated vaccines. There are four candidates in clinical development at the present time. The Walter Reed Army Institute of Research developed attenuated DENV strains.
by empirical serial passage in primary dog kidney (PDK) cells and produced vaccine candidates in fetal rhesus lung cells. Tetravalent formulations of these attenuated vaccine candidates have been evaluated in Phase 1 and Phase 2 clinical trials conducted by the Walter Reed Army Institute of Research and GlaxoSmithKline (8, 27–29).

The other three candidates were developed using recombinant DNA technology which involves first the generation of a full-length DNA copy of the DENV genome. Site-specific mutations expected to affect virulence are then introduced into the DNA, and mutant full-length DNAs can then be copied in vitro to produce infectious RNA transcripts that can be used to generate mutant DENVs in tissue culture. The United States National Institute of Allergy and Infectious Diseases (NIAID) has thus derived a total of five candidate dengue vaccine viruses that have been tested in clinical trials. Two were generated by introduction of a 30-nucleotide deletion (termed Δ30) into the 3’ UTR of the DENV-4 and DENV-1 genomes (8, 27–29). These DENV-1 and DENV-4 vaccine candidates were shown to be attenuated and immunogenic in nonhuman primates. A DENV-2 candidate vaccine was developed by replacing the gene segments encoding the prM and E proteins of the DEN4Δ30 candidate vaccine with those of DENV-2. An additional DENV-3 candidate vaccine was developed by replacing the 3’ UTR of a DENV-3 wild-type virus with that of the DEN4Δ30 UTR. A third DENV-3 candidate vaccine was developed by introducing a 30 nucleotide deletion into the 3’ UTR homologous to that of the DEN4Δ30 vaccine virus and a second non-contiguous 31 nucleotide deletion, also in the 3’ UTR (30). Phase 1 trials have been conducted with “Δ30” monovalent vaccines (31), and Phase 1 trials with the tetravalent formulation were initiated in 2010.

Thai scientists at Mahidol University developed a candidate DENV-2 vaccine empirically by 53 serial passages of the virus in PDK cells, designated DENV-2 strain PDK53, which was found to be highly attenuated and immunogenic in Phase 1 and 2 clinical trials. The United States CDC determined that the attenuation mutations of DENV-2 PDK53 virus were not located in the prM or E proteins, and in collaboration with Inviragen used this genetic background to derive chimeric DENV-1, DENV-3 and DENV-4 vaccines expressing the respective prM/E genes in the context of the DENV-2 PDK53 genome “backbone” (8, 27). A tetravalent formulation is in Phase 1 clinical trials.

Finally, a candidate live vaccine was developed by Acambis/Sanofi Pasteur using the live, attenuated yellow fever virus (YFV) vaccine, 17D, as the backbone for chimeric DENV vaccine candidate. In these viral genomes, the prM and E genes from each of the four DENV serotypes, respectively, are substituted for those of YFV in the context of the genetic background of the 17D vaccine. A tetravalent chimeric YFV-DENV vaccine has been evaluated in Phase 1 and 2 clinical trials for safety and immunogenicity. Phase 2b trials to investigate protective efficacy in children began late in 2009 (8, 27) and Phase 3 trials have been in progress since late 2010.
Part A. Guidelines on manufacturing and control of dengue tetravalent vaccines (live, attenuated)

A.1 Definitions

A.1.1 International name and proper name

Although there is no licensed dengue vaccine, the provision of a suggested international name at this early stage of development will aid harmonization of nomenclature if licensure is obtained. The international name should be “dengue tetravalent vaccine (live, attenuated)”. The proper name should be the 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

A tetravalent dengue virus vaccine (live, attenuated), as defined in section A.1.1, should contain live, attenuated dengue viruses representing each of the four serotypes, or replication-competent viral vectors that express the major structural antigen genes of each of the four dengue serotypes, that have been separately prepared in cell culture. It may be presented as a sterile, aqueous suspension or as freeze-dried material. The preparation should satisfy all the specifications given below.

A.1.3 International reference materials

As the prospective vaccines are very different in type, no international reference material for a candidate live dengue vaccine is available. However, an international reference panel of human antisera against all four dengue serotypes is available from the National Institute of Biological Standards and Control, Potters Bar, England. The panel is intended to help calibrate the response to vaccines.

A.1.4 Expression of dose related to vaccine potency

Potency of a live vaccine is usually expressed in terms of the number of infectious units of virus contained in a human dose, using a specified tissue culture substrate and based on results of Phase 1 and Phase 2 clinical trials. In the case of a tetravalent dengue vaccine, potency will have to be assessed in terms of the individual titres of each of the four serotypes of vaccine virus contained in a human dose. When international reference standards for the vaccine type under production become available, the dose related to vaccine potency should be calculated against the International Standard and expressed in International Units (IU) to reduce variation between laboratories. Until then, the use of plaque-forming unit, immunofocus-forming unit or cell culture infectious dose 50%
(CCID₉₀) to express the potency and doses of vaccine can be an alternative. The
dose should also serve as a basis for establishing parameters for stability and for
the expiry date.

A.1.5 Terminology

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

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

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

**Cell culture infectious dose 50%:** the amount of a virus sufficient to
cause a cytopathic effect in 50% of inoculated replicate cell cultures, as determined
in an end-point dilution assay in monolayer cell cultures.

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

**Cell substrates:** cells used for the production of a vaccine.

**Dengue febrile illness (DFI):** the virological confirmation of dengue
virus infection in patients with two days of fever irrespective of the severity
of illness.

**Final lot:** a collection of sealed final containers of finished vaccine
that is homogeneous with respect to the risk of contamination during filling
and freeze-drying. All the final containers should, therefore, have been filled
from one vessel of final tetravalent bulk and freeze-dried under standardized
conditions in a common chamber in one working session.

**Final tetravalent bulk:** the finished tetravalent vaccine prepared from
virus harvest pools in the vessel from which the final containers are filled.

**Genetically modified organism:** an organism in which the genetic
material has been altered in a way that does not occur naturally by mating and/
or natural recombination.

**Immunofocus-forming unit:** the amount of a virus required to generate
one focus of infected cells that can be detected by dengue-specific antisera and a
counter-stain in monolayer cell cultures.

**Master cell bank:** a quantity of well-characterized cells of animal or other
origin, derived from a cell seed at a specific population doubling level or passage
level, dispensed into multiple containers, cryopreserved, and stored frozen under
defined conditions, such as the vapour or liquid phase of liquid nitrogen in aliquots of uniform composition. The master cell bank is prepared from a single homogeneously mixed pool of cells. It is considered best practice for the master cell bank to be used to derive working cell banks.

**Virus master seed:** a suspension of vaccine virus that has been aliquoted into identical vials and stored at a temperature and under conditions deemed to stabilize the virus in each container. The virus master seed is used as a source of infectious virus for the generation of each virus working seed lot.

**Neurovirulence:** the capacity of a microorganism to cause disease of the nervous system, leading to paralysis or dysfunction of the nervous system. In animal experimental settings, clinical and pathological evaluations are often carried out after intracranial inoculation of a microorganism.

**Neurotropism:** the affinity of a microorganism for, or for localizing selectively in, nerve tissue.

**Monovalent virus pool:** a suspension of single serotype of dengue virus that may be the result of one or more single harvests or multiple parallel harvests of the same virus serotype collected into a single vessel before clarification.

**Multiple parallel harvest:** a pool of harvests coming from multiple cultures that are initiated in parallel from the same ampoule of the same working cell bank infected together by the same virus suspension of the same virus working seed lot.

**Plaque-forming unit:** the amount of a virus sufficient to cause a single visible focus of infection due to cytopathic effect in a cell culture monolayer after proper staining of cells.

**Production cell culture:** a collection of cell cultures used for biological production that have been prepared together from one or more containers from the working cell bank or, in the case of primary cell cultures, from the tissues of one or more animals.

**Single harvest:** a quantity of virus suspension harvested from production cell cultures inoculated with the same virus working seed and processed together in a single production run.

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

**Virus working seed:** a quantity of virus of uniform composition, well characterized and derived from a virus master seed lot (see above) in a production cell line. The working seed lot is used for the production of a single harvest.
A.2 General manufacturing requirements

The general requirements for manufacturing establishments contained in WHO's Good manufacturing practices for biological products (32) should be applied by establishments manufacturing dengue tetravalent vaccine. Separate manufacturing areas for each of the four dengue serotypes and for tetravalent vaccine formulation may be used. Alternatively, manufacturing areas may be used on a campaign basis with adequate cleaning between campaigns to ensure that cross-contamination does not occur.

Production steps and quality-control operations involving manipulations of live virus should be conducted under the appropriate biosafety level, as agreed with the NRA and in accordance with country biosafety laws.

A.3 Control of source materials

A.3.1 Cell cultures for virus production

A.3.1.1 Conformity with WHO recommendations

Dengue viruses used in producing tetravalent dengue vaccine should be propagated in cell substrates which meet the Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (33) and should be approved by the NRA. All information on the source and method of preparation of the cell culture system used should be made available to the NRA.

A.3.1.2 Types of cell culture

Dengue vaccine candidates have been produced in fetal rhesus lung diploid cells and in continuous cell lines. For fetal rhesus lung diploid cells and continuous cells, sections A.3.1.3 and A.3.1.4 apply.

A.3.1.3 Cell banks

The use of a cell line such as fetal rhesus lung diploid cells or Vero cells for the manufacture of dengue vaccines should be based on the cell bank system. The cell seed should be approved by the NRA. The maximum number of passages or population doubling allowable between the cell seed, the working cell bank and the production passage levels should be established by the manufacturer and approved by the NRA. Additional tests may include, but are not limited to: propagation of the master cell bank or working cell bank cells to or beyond the maximum in vitro age for production, and examination for the presence of retroviruses and tumorigenicity in an animal test system (33).

WHO has established a bank of Vero cells, designated as WHO Vero reference cell bank 10-87 that has been characterized in accordance with the
WHO Requirements for continuous cell lines used for biologicals production (34). The cell bank is available to manufacturers, as is well-characterized starting material for manufacturers to prepare their own master and working cell banks on application to the Coordinator, Quality, Safety and Standards, WHO, Geneva, Switzerland (33).

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 NRA, at which point the cells are combined to give a single pool distributed into ampoules and preserved cryogenically to form the working cell bank. The manufacturer’s working cell bank is used for the preparation of production cell culture, and thus for the production of vaccine batches.

A.3.1.4 Characterization of cell banks

The cell seed (if applicable), master and working cell banks and end-of-production cells or extended cell bank should be characterized according to the WHO Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (33).

A.3.1.5 Cell culture medium

Serum used for propagating 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 (Requirements for biological substances, no. 6) (35, 36), as well as from infectious viruses.

Detailed guidelines for detecting bovine viruses in serum for establishing a master cell bank and working cell bank are given in Appendix 1 of the WHO Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (33). The principles outlined in the cell substrate recommendations should be applied as appropriate, and the guidelines for detecting bovine viruses in serum for establishing the cell banks may be applicable to production cell cultures as well. In particular, validated molecular tests for bovine viruses might replace the cell culture tests of bovine sera if agreed by the NRA. As an additional monitor of quality, sera may be examined for freedom from phage and endotoxin. Gamma irradiation may be used to inactivate potential contaminant viruses.

The sources of animal components used in culture medium should be approved by the NRA. These components should comply with current guidelines in relation to animal TSE (37, 38).
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) (39), as well as current guidelines in relation to human TSE (37, 38).

The use of human albumin as a component of a cell culture medium requires careful consideration due to potential difficulties with the validity period of albumin (which is based on the length of time for which it is suitable for use in clinical practice) in relation to the potential long-term storage of monovalent bulks of each dengue serotype. In addition, if human albumin is used, it should be tested according to the WHO Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (33).

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 NRA. Nontoxic pH indicators may be added (e.g. phenol red at a concentration of 0.002%). Only substances that have been approved by the NRA may be added.

If porcine or bovine trypsin is used for preparing cell cultures, it should be prepared, tested and found free of cultivable bacteria, fungi, mycoplasmas and infectious viruses, as described in the WHO Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (33). The methods used to ensure this should be approved by the NRA. The source(s) of trypsin of bovine origin, if used, should be approved by the NRA and should comply with current guidelines in relation to animal TSE (37, 38).

A.3.2 Virus seeds

A.3.2.1 Vaccine virus strains

The strains of DENV 1–4 viruses attenuated either by serial passage in cell cultures or by recombinant DNA technology used in the production of candidate tetravalent dengue vaccine should be thoroughly characterized. This will include historical records (such as information on the origin of the strain, cell culture passage history, method of attenuation, results of preclinical and clinical studies demonstrating attenuation, and whether the strains have been biologically or molecularly cloned prior to generation of the master seed), their genome sequence, the passage level at which clinical trials were performed, and the results of clinical studies. Only strains approved by the NRA should be used.

Strains of dengue recombinant viruses used for master and working seeds to produce vaccine candidates should comply with the additional specifications given in section A.3.2.2.
A.3.2.2 Strains derived by molecular methods

If vaccine seeds derived by recombinant DNA technology are used, and because this is a live, attenuated vaccine, the candidate vaccine is considered a genetically modified organism (GMO) in several countries and should comply with the regulations of the producing and recipient countries regarding GMOs. An environmental risk assessment should be undertaken according to Part D of these Guidelines.

The nucleotide sequence of any cDNA clone used to generate vaccine virus stocks should be determined some time prior to any further nonclinical or clinical trial. The cell substrate used for transfection to generate the virus should be appropriate for human vaccine production and should be approved by the NRA.

Pre-seed lot virus stocks derived from passaging of the primary virus stock should also be sequenced as part of nonclinical evaluation.

Viral vaccine seeds that are directly re-derived from RNA extracted from virus in order to reduce the risk of previous contamination by TSEs or other adventitious agents are considered as new vaccine seeds, and they should be appropriately characterized to demonstrate comparability with the starting virus seed.

A.3.2.3 Virus seed lot system

The production of vaccine should be based on the master and working seed lot system to minimize the number of tissue culture passages needed for vaccine production. Seed lots should be prepared in the same type of cells using the same conditions for virus growth (other than scale) as those used for production of final vaccine.

The virus working seed should have a well-defined relationship to the virus master seed with respect to passage level and method of preparation, such that the virus working seed retains all of the in vitro and in vivo phenotypes and the genetic character of the virus master seed. Nonclinical and clinical data are needed to support this relationship. Once the passage level of the virus working seed with respect to the virus master seed is established, it may not be changed without approval from the NRA.

Virus seed lots should be stored in a dedicated temperature-monitored freezer at a temperature that ensures stability upon storage. It is recommended that a large virus working seed lot should be set aside as the basic material for use by the manufacturer for the preparation of each batch of vaccine.

Full in vitro and in vivo testing for detecting adventitious agents should be conducted on either master or working seed lots.
A.3.2.4 Control cell cultures for virus seeds

In agreement with NRAs, tests on control cell cultures may be required and performed as described in section A.4.1.

A.3.2.5 Tests on virus master and working seed lots

A.3.2.5.1 Identity

The serotype of all dengue virus master seeds and working seeds should be confirmed by immunological assay or by molecular methods.

A.3.2.5.2 Genetic/phenotypic characterization

Different live dengue vaccine viruses may have significantly different properties. Such differences may influence the tests to be used to examine their genetic and phenotypic stability relevant to consistency of production. The applicable tests will be identified in the course of the nonclinical evaluation of the strains. Each seed should be characterized by full-length nucleotide sequence determination and by other relevant laboratory and animal tests, which will provide information on the consistency of each virus seed.

Mutations introduced during derivation of each vaccine strain should be maintained in the consensus sequence, unless spontaneous mutations induced during tissue culture passage were shown to be innocuous in nonclinical and small-scale clinical trials. Some variations in the nucleotide sequence of the virus population on passage are to be expected, but what is acceptable should be based on experience in production and clinical use.

For any new virus master seeds and working seeds, it is recommended that the first three consecutive consistency bulk vaccine lots be analysed for consensus sequence changes from virus master seed. The nucleotide sequence results should be used to demonstrate the consistency of the production process.

Routine nucleotide sequence analysis of bulk vaccine is not recommended.

A.3.2.5.3 Tests for bacteria, fungi, mycoplasmas and mycobacteria

Each virus master and working seed lot 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 General requirements for the sterility of biological substances (Requirements for biological substances, no. 6) (35, 36). Nucleic acid amplification techniques alone or in combination with cell culture, with an appropriate detection method, may be used as an alternative to one or both of the compendial mycoplasma detection methods after suitable validation and agreement from the NRA (33).

Seed lots should be shown to be free from mycobacteria by a method approved by the NRA. Nucleic acid amplification techniques may be used as an alternative to the microbiological culture method for mycobacteria and/or to the
in vivo guinea-pig test for the detection of mycobacteria after suitable validation and agreement from the NRA (33).

A.3.2.5.4 Tests for adventitious agents

Each virus working seed lot and/or master seed lot should be tested in cell cultures for adventitious viruses relevant to the passage history of the seed virus. Where antisera are used to neutralize dengue virus or the recombinant dengue virus, the antigen used to generate the antisera should be produced in cell culture from a species different from that used for the production of the vaccine and free from extraneous agents. Monkey and human cell cultures inoculated with the virus antibody mixture should be observed microscopically for cytopathic changes. For virus grown in monkey or human cells, the neutralized virus is tested on a separate culture of these cells. If other cell systems are used, cells of that species, but from a separate batch, are also inoculated. At the end of the observation period, the cells should be tested for haemadsorbing viruses.

Each virus master or working seed lot should also be tested in animals that include guinea-pigs, adult mice, and suckling mice. For test details, refer to section B.11 of WHO’s Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (33). Additional testing for adventitious viruses may be performed using validated nucleic acid amplification techniques.

New molecular methods with broad detection capabilities are being developed for adventitious agent detection. These methods include: (i) degenerate nucleic acid amplification techniques for whole virus families with analysis of the amplicons by hybridization, sequencing or mass spectrometry; (ii) nucleic acid amplification techniques with random primers followed by analysis of the amplicons on large oligonucleotide micro-arrays of conserved viral sequencing or digital subtraction of expressed sequences; and (iii) high throughput sequencing. These methods may be used in the future to supplement existing methods or as alternative methods to both in vivo and in vitro tests after appropriate validation and agreement from the NRA.

A.3.2.5.5 Tests in experimental animals

Tests in nonhuman primates: all vaccine candidates should be evaluated, at least once during nonclinical development, for neurovirulence in nonhuman primates, as detailed in Part B (nonclinical evaluation). Candidate vaccines that are homogeneously “dengue” in terms of genetics are not expected to be neurotropic in such a test but, where attenuation has been achieved by recombination of dengue genes with those of a different virus species that itself displays neurovirulence (e.g. dengue/yellow fever recombinants), the master seed should be tested in nonhuman primates. If these tests were not performed at the master seed level, they should be performed at working seed level.
NRAs may decide that such testing does not need to be repeated each time a novel working seed lot is derived, if results of a well-conducted monkey neurovirulence assay on the master seed lot are negative. Recent data suggest that certain small animal models for neurovirulence may serve as a surrogate for nonhuman primates, at least where viruses expressing yellow fever strain 17D genes are concerned (40). NRAs may eventually wish to consider accepting results of such studies as a surrogate for studies using nonhuman primates to evaluate neurovirulence of novel dengue vaccines.

**Test for neurovirulence:** to provide assurance that a candidate vaccine virus is not unexpectedly neurovirulent, each vaccine strain of each serotype, or a tetravalent formulation if agreed by the NRA, should be tested for neurovirulence in monkeys by inoculation of *Macaca mulatta* (rhesus), *Macaca fascicularis* (cynomolgus) or other susceptible species of monkey, in the course of preclinical evaluation.

Prior to testing for neurovirulence, the neutralizing antibody test should be used to assess the immune status of nonhuman primates to both dengue and yellow fever viruses. For further details on the test for neurovirulence see Part B.

**Tests in suckling mice:** the virulence of different vaccine candidates in mice will depend on the strains of virus and mouse. Novel vaccines that reach the clinical phase of development in many cases were tested for neurovirulence in suckling and adult mice during the preclinical phase of development.

While mice are not considered a good model for dengue, suckling and adult mice have been used to assess the neurovirulence of dengue/yellow fever recombinant vaccines (21, 41). A mouse test might be considered in order to demonstrate consistency of characteristics of dengue/yellow fever recombinant viruses during production (see section A.4.2.4.7).

A.3.2.5.6  *Virus titration for infectivity*

Each virus master and working seed lot should be assayed for infectivity in a sensitive assay in cell culture. Depending on the results obtained in preclinical studies, plaque assays, CCID<sub>50</sub> assays, immunofocus-forming unit assays or CCID<sub>50</sub> with a molecular readout such as quantitative polymerase chain reaction may be used. All assays should be validated.

A.4  **Control of vaccine production**

A.4.1  **Control of production cell cultures**

Where the NRA requires the use of control cells, the following procedures should be followed. From the cells used to prepare cultures for production of vaccine, a fraction equivalent to at least 5% of the total or 500 ml of cell suspension, or
100 million cells, should be used to prepare uninfected control cell cultures. These control cultures should be observed microscopically for cytopathic and morphological changes attributable to the presence of adventitious agents for at least 14 days at a temperature of 35–37 °C after the day of inoculation of the production cultures, or until the time of final virus harvest, whichever comes last. At the end of the observation period, supernatant fluids collected from the control culture should be tested for the presence of adventitious agents as described below. Samples that are not tested immediately should be stored at −60 °C or lower, until such tests can be conducted.

If adventitious agent testing of control cultures yields a positive result, the harvest of virus from the parallel vaccine virus-infected cultures should not be used for production.

For the test to be valid, not more than 20% of the control culture flasks should have been discarded, for any reason, by the end of the test period.

A.4.1.1 Test for haemadsorbing viruses
At the end of the observation period, a fraction of control cells comprising not less than 25% of the total should be tested for the presence of haemadsorbing viruses, using guinea-pig red blood cells. If the guinea-pig red blood cells have been stored prior to use in the haemadsorption assay, the duration of storage should not have exceeded seven days, and the storage temperature should have been in the range of 2–8 °C.

In some countries, the NRA requires that additional tests for haemadsorbing viruses should be performed using red blood cells from other species, including those from humans (blood group O), monkeys, and chickens (or other avian species). For all tests, readings should be taken after incubation for 30 minutes at 0–4 °C, and again after a further incubation for 30 minutes at 20–25 °C. The test with monkey red cells should be read once more after additional incubation for 30 minutes at 34–37 °C.

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

A.4.1.2 Tests for cytopathic, adventitious agents in control cell fluids
Supernatant culture fluids from each of the control cell culture flasks or bottles collected at the time of harvest should be tested for adventitious agents. A 10 ml sample of the pool should be tested in the same cell substrate, but not the same cell batch, as that used for vaccine production, and an additional 10 ml sample of each pool should be tested in both human and continuous simian (monkey) cells.

Each sample should be inoculated into cell cultures in such a way that the dilution of the pooled fluid in the nutrient medium does not exceed 1:4. The surface area of the flask should be at least 3 cm² per ml of pooled fluid. At least one flask of the cells should remain uninoculated, as a control.
The inoculated cultures should be incubated at a temperature of 35–37 °C and should be examined at intervals for cytopathic effects over a period of at least 14 days.

Some NRAs require that, at the end of this observation period, a subculture is made in the same culture system and observed for at least an additional seven days. Furthermore, some NRAs require that these cells should be tested for the presence of haemadsorbing viruses.

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

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

A.4.2  Production and harvest of monovalent virus
A.4.2.1  Cells used for vaccine production
On the day of inoculation with the working seed virus, each production cell culture flask (or bottle etc.) and/or cell culture control flask should be examined for cytopathic effect potentially caused by infectious agents. If the examination shows evidence of the presence in any flask of an adventitious agent, all cell cultures should be discarded.

If animal serum is used in growth medium, the medium should be removed from the cell culture either before or after inoculation of the virus working seed. Prior to beginning virus harvests, the cell cultures should be rinsed and the growth medium should be replaced with serum-free maintenance medium.

Penicillin and other beta-lactam antibiotics should not be used at any stage of manufacture. Minimal concentrations of other suitable antibiotics may be used if approved by the NRA.

A.4.2.2  Virus inoculation
Cell cultures are inoculated with dengue virus working seed at an optimal and defined multiplicity of infection. After viral adsorption, cell cultures are fed with maintenance medium and are incubated at a temperature within a defined range and for a defined period.

The multiplicity of infection, temperature range and duration of incubation will depend on the vaccine strain and production method, and specifications should be defined by each manufacturer.
A.4.2.3  **Monovalent virus harvest pools**

Vaccine virus is harvested within a defined period post-inoculation. A monovalent harvest may be the result of one or more single harvests or multiple parallel harvests. Samples of monovalent virus harvest pools should be taken for testing and should be stored at a temperature of –60 °C or below. The sponsor should submit data to support the conditions chosen for these procedures.

The monovalent virus harvest pool may be clarified or filtered to remove cell debris and stored at a temperature that ensures stability before being used to prepare the tetravalent final bulk for filling. The sponsor should provide data to support the stability of the bulk throughout the duration of the chosen storage conditions, as well as to support the choice of storage temperature.

Harvests derived from continuous cell lines should be subjected to further purification to minimize the amount of cellular DNA, and/or to treatment with DNase to reduce the size of the DNA.

A.4.2.4  **Tests on monovalent virus harvest pools**

A.4.2.4.1  **Identity**

Each monovalent virus harvest pool should be identified as the appropriate dengue virus serotype by immunological assay on cell cultures using specific antibodies, or by molecular methods (see section A.6.1) approved by the NRA.

A.4.2.4.2  **Tests for bacteria, fungi, mycoplasmas and mycobacteria**

Each monovalent virus harvest pool should be shown by appropriate tests to be free from bacterial, fungal, mycoplasmal and mycobacterial contamination. Sterility tests are specified in Part A, sections 5.2 (bacteria and fungi) and 5.3 (mycoplasmas), of the General requirements for the sterility of biological substances (Requirements for biological substances, no. 6) (35, 36).

Nucleic acid amplification techniques alone or in combination with cell culture, with an appropriate detection method, might be used as an alternative to one or both of the pharmacopoeial mycoplasma detection methods after suitable validation and the agreement of the NRA (33).

The method for testing mycobacteria should be approved by the NRA. Nucleic acid amplification techniques might be used as an alternative to the microbiological culture method for mycobacteria after validation and agreement by the NRA (33).

A.4.2.4.3  **Tests for adventitious agents**

Each monovalent virus harvest pool should be tested in cell culture for adventitious viruses by inoculation into continuous simian kidney cells, cell lines of human origin, and the cell line used for production, but from another batch. Where antisera are used to neutralize dengue virus or the recombinant virus, the antigen
used to generate the antisera should be produced in cell culture from a species that is different from that used for the production of the vaccine and that is free from extraneous agents. 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 adventitious viruses may be performed using validated nucleic acid amplification techniques.

A.4.2.4.4 Virus titration for infectivity
The titre for each monovalent virus harvest should be determined in a sensitive assay in cell culture. Depending on the results obtained in preclinical studies, plaque assays, CCID₅₀ assays, immunofocus formation assays or CCID₅₀ with a molecular readout such as quantitative polymerase chain reaction may be used.

A.4.2.4.5 Tests for host cell proteins
The host cell protein profile should be examined as part of characterization studies (33).

A.4.2.4.6 Tests for residual cellular DNA
For viruses grown in continuous cell line cells, the monovalent harvest pool should be tested for the amount of residual cellular DNA, and the total amount of cell DNA per dose of vaccine should be not more than the upper limit agreed by the NRA. If this is technically feasible, the size distribution of the DNA should be examined as a characterization test, taking into account the amount of DNA detectable using state-of-the-art methods (33), as approved by the NRA.

A.4.2.4.7 Test for consistency of virus characteristics
The dengue virus in the monovalent harvest pool should be tested to compare it with virus working seed, or another suitable comparator, to ensure that the vaccine virus has not undergone critical changes during its multiplication in the production culture system.

Relevant assays should be identified in nonclinical studies and may include, for example, virus yield in tissue culture, plaque phenotype, or temperature sensitivity. Other identifying characteristics may also be applicable.

Assays for the attenuation of dengue/yellow fever recombinants and other vaccine viruses, if appropriate, include tests in suckling mice. Intracerebral inoculation of suckling mice with serial dilutions of vaccine and yellow fever 17D is followed by the determination of the mortality ratio and survival time. The results obtained with the vaccine are compared to the yellow fever 17D control results.

The test for consistency may be omitted as a routine test once the consistency of the production process has been demonstrated on a significant
number of batches in agreement with the NRA. Where there is a significant change in the manufacturing process, the test should be reintroduced.

A.4.2.5 Storage

Monovalent virus harvest pools should be stored at a temperature that ensures stability.

A.4.3 Final tetravalent bulk lot

A.4.3.1 Preparation of final tetravalent bulk lot

The final tetravalent bulk lot should be prepared from monovalent virus pools of the four dengue virus subtypes using a defined virus concentration of each component. The operations necessary for preparing the final bulk lot should be conducted in a manner that avoids contamination of the product.

In preparing the final bulk, any excipients (such as diluents or stabilizer) that are added to the product should have been shown to the satisfaction of the NRA not to impair the safety and efficacy of the vaccine in the concentration used.

A.4.3.2 Tests on the final tetravalent bulk lot

A.4.3.2.1 Residual animal serum protein

If appropriate, a sample of the final bulk should be tested to verify that the level of serum is less than 50 ng per human dose.

A.4.3.2.2 Sterility

Except where it is subject to in-line sterile filtration as part of the filling process, each final bulk suspension should be tested for bacterial and fungal sterility according to Part A, section 5.2 of the General requirements for the sterility of biological substances (Requirements for biological substances, no. 6) (35), or by a method approved by the NRA.

A.4.3.3 Storage

Prior to filling, the final bulk suspension should be stored under conditions shown by the manufacturer to retain the desired viral potency.

A.5 Filling and containers

The requirements concerning Good manufacturing practices for biological products (32) appropriate to a vaccine should apply.

Care should be taken to ensure that the materials from which the container and, if applicable, the closure are made do not adversely affect the infectivity (potency) of the vaccine under the recommended conditions of storage.
A final filtration could be included during the filling operations to assure sterility.

The manufacturer should provide the NRA with adequate data to prove that the product is stable under appropriate conditions of storage and shipping.

A.6 Control tests on final lot
The following tests should be carried out on the final lot.

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 form and colour. In the case of freeze-dried vaccines, a visual inspection should be performed of the freeze-dried vaccine, the diluent, and the reconstituted vaccine.

A.6.1.2 pH
The pH of the final lot should be tested in a pool of final containers and an appropriate limit should be set to guarantee virus stability. In the case of freeze-dried vaccines, pH should be measured after reconstitution of the vaccine with the diluent.

A.6.1.3 Identity
Each monovalent component of a tetravalent dengue vaccine lot should be identified as dengue or recombinant virus type DENV-1, -2, -3 or -4 by immunological assay using specific antibodies or by molecular methods. The methods used for the potency assay (section A.6.1.5) may serve as the identity test.

A.6.1.4 Sterility
Vaccine should be tested for bacterial and fungal sterility according to the requirements of Part A, section 5.2 of the General requirements for the sterility of biological substances (Requirements for biological substances, no. 6) (35), or by methods approved by the NRA.

A.6.1.5 Potency
At least three containers of each tetravalent vaccine lot should be assayed for infectivity in a validated assay in appropriate cell culture. The assay should include
a working reference preparation to control the accuracy and reproducibility of the testing system. The titre of each serotype of dengue virus in the final tetravalent mixture should be determined.

A.6.1.6 Thermal stability

The purpose of the thermal stability test is to demonstrate consistency of production. Additional guidance on evaluation of vaccine stability is provided in WHO’s Guidelines for stability evaluation of vaccines (42). At least three containers of tetravalent vaccine should be incubated at the appropriate elevated temperature for the appropriate time (e.g. 37 °C for seven days) depending on the products. The geometric mean titre of infectious virus in the containers for each individual virus serotype that has been exposed should not have decreased during the period of exposure by more than a specified amount (e.g. 1 log) that is justified by production data and approved by the NRA. Titration of non-exposed and exposed containers should be carried out in parallel. A validity control reagent of each of the four virus components should be included in each assay to validate the assay.

A.6.1.7 General safety

Each filling lot should be tested for unexpected toxicity (sometimes called abnormal toxicity) using a general safety test approved by the NRA. This test may be omitted for routine lot release once consistency of production has been established to the satisfaction of the NRA and when good manufacturing practices are in place. Each lot, if tested, should pass a general safety test.

A.6.1.8 Residual moisture (if appropriate)

The residual moisture in each freeze-dried lot should be conducive to the stability of the product, and the upper limit of the moisture content should be approved by the NRA on the basis of the results of stability testing.

A.6.1.9 Residual antibiotics (if applicable)

If any antibiotics are added during the vaccine production, the content of the residual antibiotics should be determined and should be within limits approved by the NRA.

A.6.2 Diluent

The recommendations given in WHO’s Good manufacturing practices for pharmaceutical products: main principles (43) should apply to the manufacturing and control of diluents used to reconstitute live, attenuated dengue vaccines. An expiry date should be established for the diluent on the basis of stability data.
For lot release of the diluent, tests should be done for identity, appearance, pH, volume, sterility, and the content of key components.

A.7 **Records**
The recommendations of Good manufacturing practices for biological products (32) should apply, as appropriate to the level of development of the candidate vaccine.

A.8 **Samples**
A sufficient number of samples should be retained for future studies and needs. Vaccine lots that are to be used for clinical trials may serve as reference materials in the future, and a sufficient number of vials should be reserved and stored appropriately for that purpose.

A.9 **Labelling**
The recommendations of Good manufacturing practices for biological products (32) should apply, as appropriate for a candidate vaccine, 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:

- 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 strains of dengue or recombinant viruses contained in the live, attenuated tetravalent vaccine, the minimum number of infective units per human dose, the nature of any cellular systems used for the production of the vaccine, and whether the vaccine strains were derived by molecular methods;
- 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 concerning the photosensitivity of the vaccine, cautioning that both lyophilized and reconstituted vaccine should be protected from light;
- a statement indicating the volume and nature of 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 it has been reconstituted, the vaccine should be used without delay or, if not used immediately, stored at 2–8 °C and protected from light for a maximum period defined by stability studies.

A.10 Distribution and shipping
The recommendations given in Good manufacturing practices for biological products (32) appropriate for a candidate vaccine should apply.

Shipments should be maintained within specified temperature ranges and packages should contain cold-chain monitors (44).

A.11 Stability, storage and expiry date
The recommendations given in Good manufacturing practices for biological products (32) and in Guidelines for stability evaluation of vaccines (42) appropriate for a candidate vaccine should apply. The statements concerning storage temperature and expiry date that appear on the primary or secondary packaging should be based on experimental evidence and should be submitted for approval to the NRA.

A.11.1 Stability testing
Stability testing should be performed at different stages of production – namely, on single harvests, purified bulk, final bulk and final lot. Stability-indicating parameters should be defined or selected as appropriate to the stage of production. It is advisable to assign a shelf-life to all in-process materials during vaccine production – in particular stored intermediates such as single harvests, purified bulk and final bulk.

The stability of the vaccine in its final container and at the recommended storage temperatures should be demonstrated to the satisfaction of the NRAs on at least three lots of final product. Accelerated thermal stability tests may be undertaken on each final lot to give additional information on the overall stability of a vaccine (see section A.6.1.6).

The formulation of vaccine and adjuvant (if used) should be stable throughout its shelf-life. Acceptable limits for stability should be agreed with NRAs.

A.11.2 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 loss of titre. The maximum duration of storage should be fixed with the approval of the NRA 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.11.3 **Expiry date**

The expiry date should be defined on the basis of shelf-life and should be supported by the stability studies with the approval of the NRA. If the vaccine is stored at a temperature lower than that used for stability studies and intended for release without re-assay, the expiry date is calculated from the date of removal from cold storage. The expiry dates for the vaccine and the diluent may differ.

A.11.4 **Expiry of reconstituted vaccine**

For single-dose containers, the reconstituted vaccine should be used immediately. Multi-dose containers should be kept in the dark at 2–8 °C and the expiry time for use of an opened container should be defined by stability studies approved by the NRA, but should be not more than six hours.

**Part B. Nonclinical evaluation of dengue tetravalent vaccines (live, attenuated)**

B.1 **General remarks**

Nonclinical evaluation of a live dengue vaccine includes in vitro and in vivo testing that is required prior to initiation of the clinical phase of the vaccine development programme. This testing should yield information suggesting the safety and potential for efficacy of a dengue vaccine candidate. Testing may continue in parallel with the clinical phase of product development. Tests should include product characterization at each stage of manufacture (including quantification of contaminants such as cellular proteins and DNA), proof of concept/immunogenicity studies (including dose ranging in animals etc.), toxicology if required by the NRA, establishment of a test for potency to be used throughout, and safety testing in animals (see Table A2.1). These Guidelines, which are specifically aimed at nonclinical evaluation of a live, attenuated dengue vaccine, should be read in conjunction with the WHO Guidelines on nonclinical evaluation of vaccines (45).

Although there is no animal model that precisely mimics dengue disease in humans, animal models have been and are being used in studies on immunogenicity, protective activity, toxicology and safety. Animal models were briefly reviewed at the time of preparing these Guidelines to highlight the latest developments and to provide a better understanding of their use in vaccine development.
Table A2.1
Nonclinical evaluation of dengue vaccines

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<td>Product characterization</td>
<td>Product risks are appropriate for the anticipated use.</td>
<td>Mutations in the genome may impact infection efficiency and growth capacity in different cell types, including cells of a monocyte lineage. Virus structural protein profiles; serotype identity; consistency of the manufacturing process; genetic stability of vaccine candidates.</td>
</tr>
<tr>
<td>Process development, quality control and quality assurance</td>
<td>Process meets all good manufacturing practice standards.</td>
<td>Sources of all media, cells and seed viruses; purification and virus concentration procedures; sources of all animal sera used to cultivate viruses and cells; demonstrated efficiency of purification processes; titration of virus dose; safety of excipients; standardized laboratory assays to measure immunogenicity, etc.</td>
</tr>
<tr>
<td><strong>In vivo nonclinical evaluation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immunogenicity and protective activity in an animal model</td>
<td>Demonstrate that the vaccine can protect from some aspect of dengue infection; estimate dose range for humans.</td>
<td>DENV live, attenuated vaccines are immunogenic in nonhuman primates; candidate should induce limited viraemia and should protect against viraemia following wild-type DENV challenge in nonhuman primates. Interference between DENV serotypes may be evaluated in mice or nonhuman primates, but data may not always correlate with data from humans.</td>
</tr>
<tr>
<td>Toxicity and safety</td>
<td>Product risks are appropriate for the anticipated use.</td>
<td>Focus on unexpected consequences of the effect of the vaccine dose and direct effects due to vaccine virus replication and tissue tropisms. The evaluation includes scoring and statistical analysis for histopathological lesions and clinical signs between treatment and control groups.</td>
</tr>
</tbody>
</table>
B.2 Product development and characterization

It is critical that vaccine production processes are standardized and controlled to ensure consistency of manufacture in support of nonclinical data suggesting potential safety and efficacy in humans. This is a prerequisite for entering the clinical trial phase.

Each of the attenuated virus candidates in the tetravalent dengue vaccine formulation should be characterized to define as far as is practical the critical genetic markers of attenuation and phenotypic markers that suggest that the genome of a vaccine virus has remained stable following tissue culture passage. Each vaccine virus should also be evaluated to determine whether the genetic basis of attenuation is stable enough to reduce the risk of reversion to virulence, either during manufacture or during replication in a vaccinee, using available in vivo and in vitro approaches. To this end, laboratory and animal studies should define genetic changes in the virus genome.

Phenotypic markers may be useful for detecting reversion events and to differentiate vaccine strains from wild-type virus strains in epidemiological surveillance following human immunization.

Qualification of each attenuated vaccine strain should include obtaining the consensus nucleotide sequence of the entire genome of the vaccine candidate, using the consensus nucleotide sequence of the genome of the parent virus as a comparator. This is essential for documenting the mutations in the vaccine virus genome that may correlate with its attenuated phenotype. It is also good practice to document any in vitro phenotypes of vaccine viruses that might serve as indicators of the stability of the mutations that differentiate the vaccine virus from its virulent parent. Such markers include, but are not limited to, plaque size, replication efficiency in mosquito vectors, induction of viraemia in nonhuman primates, suckling mouse neurovirulence, virulence in any other animal model, and temperature sensitivity (4, 22, 46–48). Developers should bear in mind that consensus genome sequencing is unsuitable for identifying minor or quasi-species genomes in a vaccine seed or batch (6).

B.3 Nonclinical immunogenicity and protective activity

Assessment of innate and adaptive immune responses in animals provides evidence that the dengue vaccine has replicated in the host, at the very least. Animals, particularly mice, have also been valuable for assessing the various elements of the immune response to DENV. Although there is no specific immune correlate of protection, antibodies directed against the virus E protein neutralize the virus and have been shown to protect animals when actively induced by experimental vaccines or when passively administered prior to challenge. On the basis of the accumulated data, it is generally accepted that protection in humans
should require a DENV-specific neutralizing antibody response. However, a
correlation between the titre of neutralizing antibodies in serum, as determined
in an in vitro neutralizing antibody assay (e.g. PRNT50), and protection has not
been established for any of the four serotypes of virus.

While protective activity in an animal model does not necessarily predict
the protective effect in humans, it provides useful information regarding the
potency of the vaccine.

The immune response to or protective activity of each of the four
serotypes in a tetravalent DENV vaccine should be assessed, including the
quality of response and any potential virological/immunological interference
between types.

B.4 Nonclinical toxicity and safety

B.4.1 Considerations

General guidance on the nonclinical safety assessment and design of preclinical
studies that apply to dengue vaccines is provided in the WHO Guidelines on
nonclinical evaluation of vaccines (45). The term “toxicity” is generally associated
with the untoward consequences of the administration of a nonreplicating
medicine or biological that relate to its direct dose-dependent effect in the test
animal. Thus toxicity studies entail the careful analysis of all major organs, as well
as tissues near to and distal from the site of administration, to detect unanticipated
direct toxic effects typically of a drug or nonreplicating biological agent over a
wide range of doses, including doses sufficiently exceeding the intended clinically
relevant amount of dose. It is generally expected that, if a live, attenuated vaccine
does not replicate in the test animal, direct toxic effects are very unlikely to be
detected. For live vaccines the emphasis is on the demonstration of nonclinical
safety as a consequence of vaccine virus replication.

Nonclinical safety studies of live vaccines should be required for live,
attenuated vaccines in certain stages of development. Such studies are designed
with the primary purpose of demonstrating that the vaccine(s) is less “virulent”
in the animal host than comparable wild-type viruses, and that the vaccine does
not exhibit any unexpected harmful tissue tropism and damage or the capacity
to elicit a harmful immune response. There is no animal model that replicates
human dengue disease adequately (see sections B.2.1 and B.2.2). However,
nonhuman primates and mice may provide useful information for characterizing
the viruses (see sections B.4.2 and B.4.3). The design of preclinical safety studies
should reflect route and frequency of administration, as proposed in the protocol
to support clinical trials (45).

If the live, attenuated DENV vaccine is intended to be used to immunize
women of childbearing age, developmental/reproductive toxicity studies should
be performed according to WHO guidelines (45).
B.4.2  Assessment in the nonhuman primate

B.4.2.1  Neurovirulence and neurotropism in nonhuman primates

The consensus of current opinion is that all live dengue vaccines should be tested once for neurovirulence. If any vaccine virus strain is determined to be neurovirulent to nonhuman primates on the basis of neurovirulence testing, neurotropism in nonhuman primates via the clinical or peripheral inoculation route should also be evaluated as part of the nonclinical safety study.

At this time, the most well-established model for vaccine neurovirulence is the nonhuman primate, which has historically been used to evaluate new seeds of yellow fever vaccines (17D substrains 17D204- or 17DD-derived) and live polio vaccines. Novel rodent (hamster and mouse) models for yellow fever vaccine virulence are currently under development. A rodent model could eventually be considered in place of nonhuman primate testing (40) (see section A.3.2.5.5.1).

Involvement of the central nervous system in cases of dengue fever and dengue haemorrhagic fever has usually been diagnosed as secondary to vasculitis with resultant fluid extravasation. The rarity of reports of patients with dengue encephalitis suggests that the virus does not typically cross the blood–brain barrier and infect neuronal cells (49). However, since dengue vaccine viruses are genetically altered compared to their wild-type parent viruses, it is advisable to ensure that candidate vaccines have not acquired a neurotropic phenotype as an unintended consequence of the attenuation process. This is a particular concern with regard to dengue vaccine viruses that contain yellow fever 17D chimeric genomes, and it would be of similar importance in the future if novel dengue vaccines are derived from the genomes of any other known neuropathic viruses. This evaluation could be done once at an early stage of development, using a master seed or working seed lot of the vaccine. NRAs would need to decide whether each component of the tetravalent formulation needs to be tested separately for the property of neurovirulence or whether the tetravalent formulation could be tested initially, in which case no further testing of the individual vaccines would need to be done if results of the initial tests were within predefined specifications.

Testing for neurovirulence in the nonhuman primate model via the intracerebral inoculation route should follow the WHO recommendations for neurovirulence testing of yellow fever vaccines (50, 51) as appropriate (see a brief procedure below).

Groups of at least 10 monkeys, determined to be non-immune to DENV and YFV prior to inoculation with the DENV master seed, should be inoculated intracerebrally in the frontal lobe. A control group of 10 monkeys, also demonstrated to be non-immune to DENV and YFV, should receive yellow fever 17D. All monkeys should be observed for 30 days for signs of encephalitis, prior to necropsy. If the number of monkeys, the observation period and/or
time-point(s) for necropsy for histological examination are different from these recommendations, they should be justified and agreed with the NRA. Clinical scores, and the scores of histological lesions in the central nervous system, should be recorded. An advanced histological scoring method such as automated image analysis (52) may be implemented to provide quantitative assessment of virus-induced histopathology in brain tissues if the method has been properly validated and is acceptable to the NRA. The overall mean clinical and histological scores of the test group should not exceed the scores of the yellow fever vaccine control group. The significance level in statistical difference between test and control groups should be agreed by the NRA.

B.4.2.2 Viraemia in nonhuman primates

Nonhuman primates, humans and mosquitoes are the only natural hosts of DENV (4, 6, 8). Nonhuman primates have been widely used to evaluate replication and immunogenicity of candidate dengue vaccines (3, 5, 10). Primary infection of macaques with wild-type DENV results in moderate lymphadenopathy and a robust immune response (4, 6). The nonhuman primate model has traditionally been used as an important guide for selecting vaccine strains for further development. In such studies, reduced peak titres and duration of viraemia induced by a candidate vaccine, compared to those induced by the non-attenuated parent virus, is often – but not always – a correlate of attenuation. Consequently, if a dengue vaccine candidate causes viraemia in nonhuman primates comparable to that caused by its wild-type parent virus, the vaccine developers may wish to consider discontinuing further development.

B.4.3 Assessment in mouse models

DENV infection has been studied in many different mouse models (4, 10–13). When appropriate, a mouse model may be selected to evaluate the potential of a candidate vaccine to cause disease in comparison to its wild-type parent virus. In such an experiment, the titres of virus in blood, spleen, liver, lymph nodes, lungs, brain and other tissues at various post-infection time-points can be evaluated (4). The AG129 interferon receptor-deficient mouse will support replication of selected DENVs of all serotypes (22, 48). A DENV-2 strain adapted to replicate in the AG129 mouse induces a physiologically relevant disease in that strain (10). At present, the AG129 mouse seems suitable for safety studies, but NRAs should be aware of the pitfalls of interpreting results since these animals do not possess an intact innate immune response. For this same reason, as mentioned earlier, it would not be advisable to use AG129 mice for classic toxicology studies. Other inbred mouse strains with genes knocked out are under investigation as models of DENV infection and disease. One or more of these may have applicability to vaccine development in the future.
B.4.4 DENV replication in vector mosquitoes

Transmission of DENV to arthropod vectors from humans is essential in maintaining the virus in nature. As noted previously, none of the DENV live, attenuated candidate vaccines studied to date induces a viraemia in vaccinees that is sufficient in magnitude to infect feeding mosquitoes (6, 15, 19). Further, if mosquitoes are infected with dengue vaccines, the viruses do not replicate sufficiently to permit transmission of the virus. For these two reasons, Ae. aegypti mosquitoes are not expected to transmit dengue vaccine viruses (6, 18, 19). As a measure of attenuation and safety, future novel candidate vaccines should be shown to have reduced ability to replicate and disseminate in Ae. aegypti mosquitoes that have been infected in a controlled laboratory setting, using parent strains as controls (6, 16, 18, 53).

B.5 Environmental risk

The primary environmental risks of live dengue vaccines relate to their capacity to be spread from human to human by vector mosquitoes, and the risk that prolonged or repeated cycles of replication in mosquitoes could permit reversion to virulence. As previously noted, live vaccines currently under development have been shown to replicate poorly both in vaccinees and in mosquitoes, such that the risk for transmission by the mosquito vector is very low, if any risk exists at all (14–16, 18, 19, 54). These factors should markedly reduce the chance that any of these vaccines could revert in mosquitoes to a virulent phenotype when used in a mass vaccination campaign in an endemic area. In addition, genetic stability during multiple sequential passages in mosquitoes has also been demonstrated for most existing live dengue vaccine candidates. For future candidate novel live vaccines, similar studies would need to be done.

Some investigators have recently raised a concern regarding live dengue vaccines, suggesting that vaccine viruses might revert to virulence in mosquitoes via intragenic recombination with endogenous wild-type flaviviruses. Such a phenomenon would seem to be highly unlikely due to the factors noted above plus the controversial question of whether flaviviruses are able to undergo recombination at all, even under ideal conditions in vitro.

Guidelines for live dengue vaccines derived by recombinant DNA technology are described in Part D below.

Part C. Clinical evaluation of dengue tetravalent vaccines (live, attenuated)

C.1 General considerations for clinical studies

The following should be read in conjunction with: WHO’s Guidelines on clinical evaluation of vaccines: regulatory expectations (55); and Guidelines for the clinical evaluation of dengue vaccines in endemic areas (56).
C.1.1 Objectives of the clinical development programme

The clinical evaluation of a candidate live dengue tetravalent vaccine should document:

- the immune responses elicited by the vaccine against all four dengue serotypes;
- vaccine efficacy in the prevention of DFI of any severity caused by any of the serotype 1, 2, 3 and 4 viruses over an appropriate minimum period of observation;
- the safety profile.

In addition, the programme should:

- gather preliminary evidence that the immediate and longer-term immune response to a candidate dengue vaccine does not predispose vaccinated individuals to develop severe DFI (e.g. including haemorrhagic manifestations and systemic shock) during natural infections;
- attempt to examine the association between neutralizing antibody titres and protection against clinical disease (referred to as a surrogate marker for efficacy in this document);
- attempt to identify a neutralizing antibody titre that predicts (in the short or longer term) protection against clinical disease (referred to as an immunological correlate of protection in this document).

C.1.2 Outline of the clinical development programme

In the initial clinical studies (i.e. Phase 1 studies) it is expected that relatively small numbers of healthy adults are vaccinated with investigational vaccine formulations and that the primary focus is on assessing safety. These studies may include exploration of immune responses to ascending doses of the four DENV serotypes when administered alone and in combination.

The subsequent clinical studies (i.e. Phase 2 studies) should be designed to select a dose of each DENV serotype for use in the tetravalent candidate vaccine formulation and to identify an appropriate primary immunization schedule for further study.

It is not currently possible to license candidate dengue vaccines only on the basis of safety and immunogenicity data because there is no established surrogate marker for protection and, hence, no immunological correlate of protection has been identified.

Therefore, candidate tetravalent dengue vaccines should be evaluated for protective efficacy against DFI.
Sponsors may decide to conduct at least one preliminary study of protective efficacy (sometimes referred to as a Phase 2b study) in order to identify a final candidate vaccine and immunization schedule for further study. Alternatively, depending on the data already accumulated (e.g. based on demonstration of a robust neutralizing antibody response), sponsors may consider it appropriate to omit such a study.

The selected candidate vaccine should be evaluated in at least one adequately sized study of protective efficacy (i.e. Phase 3 study) that compares numbers of cases of virologically confirmed DFI (see section C.3.3.5) between groups of vaccinated and unvaccinated subjects. The total DFIs counted should include those due to any of the four DENV serotypes and of any degree of clinical severity that occur within a defined observation period.

Section C.3 gives more details of study designs and populations to be enrolled in studies conducted at each phase of development.

C.2 Immunogenicity
C.2.1 Measurement of immune responses to vaccination

Current evidence suggests that neutralizing antibody against each DENV serotype is likely to be the best surrogate marker for efficacy.

It is recommended that the methodology for determination of DENV serotype-specific neutralizing antibody titres should follow WHO guidelines for the plaque-reduction neutralization test (PRNT) (57). If alternative methods for determining neutralizing antibody (e.g. high throughput microneutralization assays) are developed, these should be validated against the PRNT.

Vero cells for dengue PRNT are available from the National Institute of Biological Standards and Control, England. Serum neutralization titres should be expressed in IUs calibrated against the reference panel of human antisera for dengue (see section A.1.3). In-house virus strains may be used.

An assessment of neutralizing antibody titres against each of the four serotypes of DENV is required. Additional testing against a range of strains of those serotypes, including recent wild-type isolates, is encouraged. This would be valuable information to obtain due to worldwide strain diversity and because neutralizing antibody titres against specific isolates will be variable. Such additional assays could be applied to subsets of sera collected from vaccinees who have been selected randomly, or on the basis of a scientific justification (e.g. to select sera known to cover a range of neutralizing antibody titres against the reference or in-house strains).

The assay of DENV-specific antibody other than neutralizing antibody (e.g. IgM and IgG ELISA) may be of interest but is not considered to be essential for the assessment of potential vaccine efficacy.
It is considered unlikely that data on cell-mediated immunity will provide an immunological correlate of protection. However, the exploration of cell-mediated immunity is encouraged since specific cell-mediated immunity assays may be useful for the assessment of immunological memory and durability of protection. Assessments of cytokine responses may assist in the evaluation of vaccine safety and may provide some indication of the potential risk that vaccination could predispose subjects to develop severe DFI during subsequent natural infection (58).

C.2.2 Investigation and interpretation of immune responses to vaccination
There is no established immunological correlate of protection against any DENV serotype. In the initial clinical studies of safety and immunogenicity, including the dose-finding and regimen-finding studies, it is essential to describe fully the pre-vaccination and post-vaccination neutralizing antibody titres that are observed against each of the four DENV serotypes (see also section C.3.1). Adequate data should be generated to describe the kinetics of the neutralizing antibody response in the short term. Longer-term antibody persistence data may be collected in these and/or in later studies, as described below.

In a non-endemic population with no detectable pre-vaccination neutralizing antibody in the majority of subjects, a comparison of percentages with a detectable neutralization titre post-vaccination (which may be defined as seroconversion in such a population) should be made against each DENV serotype. The analyses should also look at proportions that seroconvert (in accordance with an appropriate definition of seroconversion stated in the protocol) to multiple serotypes (i.e. two, three or all four serotypes).

In an endemic population in which very high proportions of subjects are already seropositive for neutralizing antibody with respect to at least one dengue type, a comparison of pre-vaccination and post-vaccination geometric mean titres with respect to those types will be informative, in addition to analyses based on seroconversion rates and increments in antibody from pre- to post-vaccination.

In endemic and non-endemic populations, detailed consideration of reverse cumulative distribution curves is important. For example, it may be informative to compare percentages achieving a predefined high titre of neutralizing antibody. In protective efficacy studies, neutralizing antibody against DENV serotypes should be determined and followed over time in predefined subsets of the study population, including an assessment of antibody persistence after the protocol-defined period for the primary evaluation of protective efficacy. It is preferable that the subsets of subjects to be included in these detailed immunogenicity evaluations should be identified at the time of randomization, with stratification for age and any other factors that may have an important impact on immune responses to vaccination. In any case, the data should be analysed
according to predefined subsets. Immune responses should be determined for vaccinated and unvaccinated subjects so that the effects of background exposure to DENVs during the study period can be assessed.

Depending on the specific vaccine construct and taking into account any pertinent results of nonclinical studies, sponsors may wish to undertake some exploratory investigations of antibody against other antigens (e.g. those associated with the attenuated yellow fever virus backbone in a chimeric vaccine).

Long-term storage of sera is encouraged since future developments in the field, and/or emerging data on longer-term safety or efficacy, may point to the need for additional investigations that cannot be predicted at the time of conducting the study.

Subsets of subjects should also be identified for collection of peripheral blood mononuclear cells, taking into account feasibility issues such as the blood volumes required from different age groups to produce adequate cell numbers for study and accessibility to adequate sample processing and storage facilities.

For the analysis of the relationship between neutralizing antibody titres and protection against virologically confirmed DFI, sera should be collected at timed intervals from a substantial cohort of subjects (and preferably from the entire study population, if feasible). Once the protocol-defined double-blind observation period has been completed, the initial analysis of the relationship between immune response and protection against DFI should follow. The most likely approach would be a cohort study in which one or more measures of the immune response to vaccination are related to disease in all, or in a large subset of, immunized subjects. Further analyses using longer-term follow-up data should be planned.

The use of serology to help identify infections with dengue viruses (whether or not clinically apparent) is a separate issue that is discussed in section C.3.3.

C.3 Clinical studies
C.3.1 Phase 1 studies
The Phase 1 studies should be designed to provide an early indication of whether severe local and/or systemic adverse events may occur commonly after vaccination. These studies may also provide preliminary data on immune responses to assist in the selection of DENVs (or constructs) and doses to be included in candidate tetravalent vaccine formulations for further study.

Subjects enrolled in these initial studies should be healthy adults who are naive to flaviviruses (based on medical and vaccine history and serological studies). It is preferred that the subjects are resident in non-endemic areas so that they are not at risk of natural infection with dengue or other flaviviruses. Eligible subjects should not be in need of vaccination against other flaviviruses, at least throughout the duration of the study.
Sponsors may choose to commence studies with a monovalent vaccine (i.e. containing a single live, attenuated DENV serotype) before progressing to evaluate multivalent versions (which may include bivalent, trivalent and then tetravalent formulations) of a candidate dengue vaccine.

If a candidate tetravalent vaccine formulation elicits a much lower antibody titre to one (or more than one) DENV serotype than to others, it is important that consideration is given to modification of the vaccine (e.g. by modifying the infectious titres of serotypes), and/or the immunization schedule, due to the potential implications for safety and efficacy.

If a likely candidate tetravalent vaccine is identified, it may be appropriate for a preliminary exploration of safety and immunogenicity to be conducted in healthy adult residents of an endemic area (i.e. including subjects with evidence of some pre-existing immunity to dengue or other flaviviruses). Such a study could provide further reassurance regarding the ability of the candidate vaccine to elicit immune responses to all four DENV serotypes before progressing to studies in larger numbers of subjects.

C.3.2 Phase 2 studies
The Phase 2 studies should extend the information on safety and immunogenicity of candidate vaccine formulations. They should include studies in residents of endemic areas who are therefore at risk of natural infection with dengue and may have some degree of pre-existing immunity to one or more DENV serotypes and to other flaviviruses.

While the first data may be obtained in adults there should be a plan to move down to younger age groups in a stepwise fashion. The age range should reflect that proposed for the evaluation of protective efficacy of the tetravalent vaccine candidate. Depending on the findings of the Phase 1 studies, the first Phase 2 studies may further explore dose–response relationships. The data generated on safety and immunogenicity should be sufficient to support the selection of one or more candidate tetravalent vaccines and immunization schedules (i.e. number of doses and dose intervals) for further evaluation.

If the sponsor chooses to undertake a preliminary (i.e. Phase 2b) study of safety and efficacy, this should be of an appropriate design and of adequate size to support a robust decision regarding the vaccine formulation and schedule to be further evaluated (see section C.3.3). Even in a Phase 2b study, it is recommended that subjects should be followed up for approximately 3–5 years from the time of completion of vaccination to collect data on safety and to document antibody to DENV serotypes in subsets of each treatment group.

The total number of subjects enrolled in Phase 2 studies should be sufficient to describe at least common adverse reactions to vaccination with some degree of confidence. Therefore it is expected that several hundred subjects
should have been exposed to candidate tetravalent vaccines containing the final or near-final doses of DENVs of each serotype. If any unusual, severe or serious adverse reactions are documented, it may be appropriate for further studies to include the assessment of safety as one of the primary objectives provided that these reactions would not preclude further vaccine development.

C.3.3 Phase 3 studies

Each tetravalent candidate vaccine should be evaluated in at least one study that is of an appropriate design and adequate size to estimate vaccine efficacy. This requirement may change in the future (see section C.3.3.7).

C.3.3.1 General issues for study design

Vaccine efficacy is estimated by comparing the total numbers of virologically confirmed cases of DFI of any degree of severity, and due to any of the four DENV serotypes, between the vaccinated and unvaccinated (control) groups. The primary analysis of vaccine efficacy should be conducted at the conclusion of a protocol-defined double-blind observation period. Each study should be of sufficient size and duration to provide a robust estimate of vaccine efficacy and to provide preliminary evidence that the vaccine does not predispose recipients to develop one of the severe forms of DFI following natural infection.

Studies of protective efficacy should be performed in endemic areas where a proportion of the population is likely to have some naturally acquired immunity to one or more of the four DENV serotypes and/or other flaviviruses. It is assumed that, in most – if not all – cases, each study will evaluate a single tetravalent candidate dengue vaccine and immunization schedule. However, the study design may be adapted as necessary if more than one possible active vaccination group is to be included.

Studies that involve vaccination of a large proportion of subjects at any one study locality carry the potential to interrupt DENV transmission significantly during the observation period. The result could be a reduced likelihood of demonstrating a difference in the numbers of virologically confirmed cases of DFI between the vaccine and control groups. Consideration should be given to this possibility when designing the study.

Randomization should be performed using a centralized system. When using a 1:1 randomization ratio, the block size should be selected with the aim of enrolling approximately equal numbers in test and control groups at each of the study sites so that subjects in each group are at the same risk of developing mild and severe DFI throughout the observation period. It is also possible to consider the use of unbalanced randomization (e.g. vaccine:control = 3:2 or 2:1) provided that care is taken to ensure that the desired ratio is applied at each study
site (or geographically localized sites) and the sample size is calculated to provide adequate power.

The decision to use unbalanced randomization should take the possible advantages and disadvantages into consideration. Advantages include a larger safety database and possibly easier enrolment due to the greater chance that any one subject would receive the candidate dengue vaccine. Disadvantages include the possibility that a larger proportion vaccinated against dengue could increase the risk of achieving a reduction in DENV transmission sufficient to influence the chance of obtaining a conclusive study result.

Whenever possible, subjects randomized to the control group should receive an alternative active vaccine (i.e. not a dengue vaccine) that can be given by the same route of administration as the candidate tetravalent dengue vaccine, rather than injections of placebo. The active vaccine should be selected to provide an anticipated benefit to study participants. However, such an appropriate vaccine may not always be available and there may be no option to using placebo injections to maintain the double-blind design. In addition, if the active control vaccine cannot be given at the same schedule as the candidate dengue vaccine, then placebo injections may need to be used within the schedule, as necessary, to maintain a double-blind design.

If the active control vaccine has a different presentation or appearance from those of the candidate dengue vaccine, study personnel who administer the vaccinations should not have any other involvement in the conduct of the study. Vaccine recipients should not be allowed to observe preparation of the vaccines for injection (e.g. any reconstitution steps that may or may not be necessary) to avoid the risk of their sharing this information and so identifying themselves with one of the study groups.

If the use of a placebo control is necessary to achieve a double-blind design, the protocol could plan to administer a suitable licensed vaccine to all subjects in the study (i.e. those who do and who do not receive the candidate dengue vaccine) at some time after completion of the assigned study treatments and during the double-blind follow-up period. In this way, all study subjects can derive some potential benefit from participation in the study without compromising the study’s integrity.

It is expected that several different production lots of vaccine will be used during protective efficacy studies. The decision whether a formal lot-to-lot consistency study should be built into the protocol, with the specific aim of comparing safety and immunogenicity between subjects who receive different lots (usually three of the total used) according to predefined criteria, must be made on a case-by-case basis. If such a formal comparison is to be made, additional measures will be needed to ensure that adequately sized subsets of subjects are randomized to receive each of the vaccine lots identified for this comparison.
C.3.3.2 Study location and duration

The geographical areas selected for study should have background rates of DFI that are sufficient to provide enough cases in the control group during the observation period to facilitate the estimation of vaccine efficacy. In order to assess background rates, efficacy studies should be preceded by the collection of epidemiological information to document the expected incidences of DENV serotype-specific DFI, and all DFI, preferably over several years. The data should include information on seasonality of disease to identify periods of transmission and case demographics (e.g. age and sex), so that the populations at highest risk of DFI can be targeted for enrolment.

There should also be an assessment of the likely extent of exposure of the population to other species of flaviviruses at potential study sites, because such exposure may confound the interpretation of dengue-specific serological data and may possibly affect the clinical course of DFI. This assessment should take into account any available epidemiological data, serological studies, and information on rates of vaccination against other flaviviruses.

Study sites should be endemic for dengue disease. Site selection should be based on the information collected prior to study initiation regarding the expected number of cases of dengue within the study population each season during the observation period, which would probably range from one to three years from the time of the first vaccination. Nevertheless, even if the study is conducted over several seasons and at geographically dispersed study sites, there may not be sufficient numbers of cases of DFI to support an estimation of serotype-specific vaccine efficacy for some or all of the four serotypes. Additional evidence for protective efficacy against individual DENV serotypes should be sought from post-licensure (i.e. effectiveness) studies, as discussed in sections C.3.3.7 and C.4.

There should be a plan for follow-up of subjects for safety and efficacy for at least 3–5 years from the time of completion of primary vaccination. During this period it is possible that an efficacious dengue vaccine may be offered to subjects originally assigned to the control group with potential implications for interpretation of the data that can be collected (see section C.3.3.7).

C.3.3.3 Study population

Since protective efficacy studies should be performed in endemic areas, there is a need to consider that the ultimate target group for vaccination may range from a subgroup (e.g. a specific age range) to the entire population. There are likely to be concerns regarding the inclusion of infants in protective efficacy studies because of the possible risk of DFI that has been reported in association with waning maternal antibody against one or more DENV serotypes and the unknown effects of vaccination in the presence of maternal antibody.
Therefore, it is expected that protective efficacy studies would probably exclude subjects aged under one year but should enrol children across a wide age range subject to satisfactory results from the safety and immunogenicity studies. Section C.3.3.7 considers bridging the observed vaccine efficacy to populations that were not included in efficacy studies.

C.3.3.4 Objectives, end-points and analyses

The primary objective of an efficacy study is to estimate vaccine efficacy against DFI. The primary analysis should seek to demonstrate superiority for the vaccinated group versus the control group in terms of the total numbers of cases of virologically confirmed DFI in subjects who have been fully vaccinated in accordance with the protocol and have been followed up for the required time with no major protocol deviations. In this analysis, counting of cases should commence from a designated time-point after the last dose of protocol-assigned doses has been administered.

Vaccine efficacy is estimated by comparing the total numbers of virologically confirmed cases of DFI (i.e. summation of cases due to any DENV serotype and of any degree of severity) that occur in vaccinated and unvaccinated (control) groups during a protocol-defined double-blind observation period. Vaccine efficacy should be calculated using the standard formula VE (%) = 100 × (1– r1/r0) (where VE = vaccine efficacy, r1 = incidence rate in the vaccine group, and r0 = incidence rate in the control group).

The assessment of DENV serotype-specific vaccine efficacy should be a major secondary objective and should be the subject of a planned secondary analysis. It is not expected that the study would be powered to support a formal statistical analysis of DENV serotype-specific efficacy.

The statistical analysis plan should explain how multiple episodes of DFI in any one study participant will be handled in the analyses.

The following secondary analyses are suggested for inclusion in the study protocol (although some of the data needed to complete these analyses may not become available until some time after completion of the double-blind observation period that precedes the primary analysis):

- efficacy based on counting all DFI that occur after administration of the first dose of protocol-assigned treatment;
- efficacy in all vaccinated subjects regardless of protocol deviations (including those with incomplete vaccination courses and missing data);
- efficacy according to pre-vaccination flavivirus serological status, which might be determined in a randomized subset of enrolled subjects who are followed serologically;
- efficacy according to severity of virologically confirmed DFI (with adequate protocol definitions);
- efficacy that includes prevention of “possible” or “probable” dengue infection (e.g. applied to patients in whom serology is used as the basis for dengue diagnosis without a virologically confirmed diagnosis). The justification for this secondary analysis is based on expectation that a dengue vaccine may reduce the viraemia, so making it more difficult to detect in patients who may also have abbreviated clinical signs and symptoms. Thus, serological secondary end-points may help assess overall efficacy, assuming that serological assays are equally sensitive and specific to DENV (but not to individual serotypes) in detecting dengue infection in vaccine and control groups; or
- the effect of vaccination on the duration of hospitalization and/or need for specific interventions to manage the clinical illness.

If more than one study of protective efficacy is performed with a single candidate vaccine (e.g. perhaps covering different geographical regions) using the same or a very similar study protocol, it may be appropriate to predefine a pooled analysis of the data. This pooled analysis could provide additional insight into serotype-specific vaccine efficacy and the risk of severe DFI in vaccine and control groups.

Each study should have in place a data and safety monitoring board consisting of persons with no involvement in study conduct and analysis and including a statistician. The charter of the data and safety monitoring board should enable it to unblind treatment assignments as necessary, and to recommend that enrolment is halted or the study is terminated on the basis of predefined criteria designed to protect subjects from harm. In addition, studies may include one or more planned interim analyses with predefined stopping rules.

C.3.3.5 Case definitions
The case definitions for the primary and various secondary analyses, with details of the criteria to be met, should be stated in the protocol and should be in accordance with the latest WHO recommendations (2).

Clinical diagnosis: the most commonly diagnosed form of clinically apparent dengue virus infection is characterized by the sudden onset of fever lasting at least two, and up to seven, days. Fever is commonly accompanied by severe headache, pain behind the eyes, gastrointestinal symptoms, muscle, joint and bone pain and a rash. These cases are usually self-limiting and result in complete recovery.
For the purposes of classification of cases it is important to characterize the severity of each DFI. The criteria used to assess severity should be those described by WHO that are current when the protocol is finalized (WHO/HTM/NTD/DEN/2009.1 (2) at the time of preparation of these Guidelines). These criteria should be used to determine the features of DFI that are captured in the case report form.

**Virological diagnosis:** all methods used for the virological component of the case definition should be fully validated. Virological confirmation of the clinical diagnosis can be based on direct detection of dengue viraemia by isolation. However, the use of alternative virological methods to confirm the diagnosis (e.g. detection of NS1 to demonstrate the presence of DENV and the use of reverse transcription-polymerase chain reaction (RT-PCR) to detect dengue viraemia and/or determine the serotype) is acceptable. The standardization of viral diagnostic methods is encouraged. Every effort should be made to conduct testing in one or a small number of designated central laboratories with appropriate expertise.

Obtaining specimens to attempt virological confirmation of the diagnosis should be triggered by a set of clinical features that are laid down in the study protocol and that aim to identify all potential cases of DFI of any severity as early as possible, taking into account the observation that virological diagnostic methods (including virus isolation and PCR-based assays) are more sensitive during the first five days of infection.

**Serological diagnosis:** commercial and/or in-house serological assays (e.g. enzyme immunoassay, immunofluorescence and virus neutralization tests) may be performed on paired acute and convalescent sera. The results may be used to identify possible cases of DFI in which a virological diagnosis was not confirmed, and the numbers may be compared between vaccinated and control groups in an additional secondary analysis of vaccine efficacy.

Nevertheless, although an acute primary infection may be implied from a rise in IgM levels during the first two weeks post-infection, such data need to be interpreted with considerable caution. For example, the IgM response to acute infection may be blunted in vaccinated subjects and in those infected previously by wild-type DENV or by another flavivirus. Depending on the timing of the illness, the results may also be confounded by the fact that IgM and IgG responses may reflect recent dengue vaccination rather than acute infection with wild-type dengue. In this regard, the ratio of IgM and IgG may assist in the differentiation of primary and secondary infections.

The interpretation of serological data is also complicated by cross-reacting antibody among flaviviruses. In those instances where cross-reaction with other flaviviruses does not occur, a fourfold or greater rise in dengue neutralizing antibodies makes it possible to attribute recent infection to a dengue virus presumptively – but not definitively.
C.3.3.6  Case detection and description

It is essential that there is adequate surveillance to detect any possible case of DFI as early as possible in order to optimize the chances of virological confirmation of the diagnosis. The surveillance mechanisms (e.g. including arrangements for periodic home visits or telephone calls, and involvement of hospitals serving the study catchment areas) should be tested before the study is initiated at each study site. It is essential that study subjects are educated regarding the need to contact, or directly present to, the designated study health-care facilities whenever they develop signs or symptoms that may be indicative of DFI. A checklist of these signs and symptoms should be provided to all study participants at the time of enrolment.

In addition, measures should be in place to follow each possible case of DFI for any change in disease course (e.g. progression from mild to severe DFI, onset of complications) and to document the outcome, including the time to recovery or death. In case of death before collection of specimens or in the absence of virological confirmation of the diagnosis, permission should be sought to perform a postmortem examination or, if this is refused, to at least obtain a specimen for virological examination using needle puncture of the liver.

In some study sites that are otherwise considered suitable, it may not be possible to identify a local health-care facility willing to participate in the study. These sites should not be initiated unless there is at least agreement from local health-care providers to notify study staff of possible cases of DFI within a time frame that is sufficient to allow for specimens to be collected and transported for virological diagnosis. Subjects should carry a study participant card, with contact names and numbers, to ensure that study personnel are alerted and can arrange for the collection of all the necessary clinical data and the transport of specimens for virological diagnosis.

Despite taking the steps described, there will still be some cases of possible DFI that are not confirmed virologically and for which serological testing is inconclusive. In addition, some subjects may not comply with the study requirement to present to a designated health-care facility when they have signs and symptoms indicative of a possible DFI, or they may be so ill that they are immediately admitted to a hospital not directly participating in the study and/or may die without notification of study personnel in time to collect data and specimens. It is important that as much information as possible is collected on these cases whenever and however they come to light, and that they are taken into account in a “worst-case scenario” analysis of vaccine efficacy that counts all cases (proven and unproven and regardless of protocol deviations).

C.3.3.7  Need for additional studies of efficacy

Once the efficacy of at least one candidate dengue vaccine has been satisfactorily demonstrated (and it is perhaps already licensed and introduced into the routine
vaccination programme in at least one country) there will be a need to reassess the content of clinical development programmes for other candidate dengue vaccines. For example, depending on the licensed dengue vaccine(s) available and the data that have been generated during their development, it may or may not be feasible or considered necessary to conduct studies that include an unvaccinated control group with subsequent candidate tetravalent vaccines.

It is not currently possible to make a definitive recommendation regarding what could or should be required in this scenario, since much will depend on the findings reported from the first completed efficacy study of a candidate vaccine or from ongoing studies with other candidate vaccines. Some pertinent issues are discussed below.

Once one or more dengue vaccine(s) has been licensed and introduced into the routine vaccination programme in one or more countries, the inclusion of an unvaccinated group in subsequent studies in these (and possibly other) countries may be considered unethical. However, studies that include an unvaccinated group may be feasible in some regions after approval of the first vaccine where there is appropriate justification and if approved by the NRA. In such a case, standard care and protection of study subjects should be provided as appropriate.

Efficacy studies using an unvaccinated control group and relative efficacy studies (i.e. studies in which the test vaccine is compared to a licensed vaccine) may not be feasible once a dengue vaccine has been introduced into the routine vaccination programme in a country or region, because this may reduce the incidence of DFI to levels that are too low to permit the estimation of vaccine efficacy from further studies of feasible size and duration.

However, if there remains considerable uncertainty about vaccine efficacy against one or more DENV serotype(s), efficacy studies that include an unvaccinated control group might be possible in regions where such serotype(s) are predicted to predominate.

It is very possible that immunological correlates of efficacy for each of the four serotypes of dengue virus cannot be established by analysis of early results of the first efficacy trials. Until surrogates and correlates of protection have been established, it may not be possible to determine the efficacy of a novel live vaccine by conducting a head-to-head comparison of its immunogenicity to that of a licensed live vaccine (i.e. in a bridging study). It may also be problematic to use bridging studies to support the extrapolation of efficacy observed with live virus vaccines to other types of dengue vaccine (e.g. killed virus vaccines, DNA vaccines, subunit vaccines). However, there may be no alternative to the use of bridging studies because of the factors described above.

Before resorting to bridging studies, there should be a careful scientific evaluation of the arguments for and against extrapolation of the efficacy observed
for a particular vaccine to populations that differ in character from the population in which the efficacy study was actually performed. Examples include populations that differ in age, risk for severe dengue, ethnicity, and/or prior or concurrent exposure to other flaviviruses.

C.3.3.8 Documentation of safety during pre-licensure studies

The routine monitoring of safety during all pre-licensure clinical studies should follow the usual principles taking into account issues relevant to live, attenuated vaccines. In addition to providing study-specific safety data, there should be an analysis of safety data pooled across all study groups that received the final selected vaccine formulation.

There is a particular need to assess whether DFI (which could be of any degree of severity, including very mild illness) may be caused by vaccine strains. In all cases of fever or other dengue-like signs or symptoms that occur following vaccination during clinical studies, it is essential that laboratory investigations are undertaken to determine whether or not the vaccine is responsible. Since the results will not always allow for a clear judgement of relatedness to vaccination, the protocol should provide criteria for ranking causality.

Low-grade and very transient fevers are to be expected and have been routinely observed in a small fraction of vaccinees after exposure to live dengue vaccines. NRAs will have to judge on a case-by-case basis whether the incidence, duration and/or severity of febrile episodes that cannot be ascribed to intercurrent illness that is observed during early studies in non-endemic areas are unacceptable. In these studies the level of vaccine viraemia (determined by RT-PCR and/or by direct culture methods) and level of vaccine virus NS1 antigenaemia should be determined in each vaccinee at one or more time-points post-vaccination in order to establish an average profile for the novel live vaccine under study. Levels higher than the predetermined average levels of vaccine virus or NS1 protein in blood detected during a febrile episode could be taken as evidence in favour of a direct causative role for the vaccine. During later studies in endemic areas there is a need to distinguish vaccine-associated from naturally occurring DFI, in addition to the other issues noted above.

There is a risk that vaccination could predispose recipients to developing a severe form of DFI. The risk may increase with time elapsed since vaccination in relation to waning titres of vaccine-induced antibodies in subjects who have not been naturally boosted in the interim period. The monitoring and investigation of all subjects who develop signs or symptoms potentially indicative of DFI during pre-licensure studies in endemic regions should provide a preliminary assessment of this risk. If no undue risk is identified and the vaccine is licensed, it is essential that there is adequate follow-up of study subjects together with further assessment of the risk in the post-licensure period (see section C.4).
The total safety database derived from all pre-licensure studies should be sufficient to describe uncommon adverse reactions. It is desirable to rule out events that occur at a frequency greater than 1:1000 vaccinees.

On the basis of the considerations outlined in section C.3.3.7, it may be that vaccines that are developed subsequent to the approval of the first vaccine(s) will not be evaluated in pre-licensure studies of protective efficacy, with implications for the size of the safety database. NRAs will need to assess numbers that would constitute an adequate safety database before initial licensure. In addition, NRAs may make specific recommendations regarding the method of data collection, classification and scoring of severity of adverse events that are captured, as well as the post-vaccination duration of the safety data collection (i.e. after each dose and following the last dose of a course).

C.4 Post-licensure investigations

There is a need to ensure that adequate surveillance is in place and is maintained to detect adverse reactions during the post-licensure period, in accordance with requirements of the countries in which approval has been obtained.

The need for, and the design and extent of, specific studies of safety and/or effectiveness following approval of a dengue vaccine should be given careful consideration by sponsors and NRAs. There will be a clear need to try to collect information on the following:

- long-term evaluation of breakthrough cases of DFI, to detect any waning of protective immunity against one or more DENV serotypes and the possible need for booster doses (there may be considerable practical difficulties in collecting reliable data in some settings; therefore it is recommended that sponsors and NRAs discuss ways in which such data could be obtained at least in some areas/regions);
- persistence of the immune response to vaccination (e.g. based on serial measurements of neutralizing antibody and detection of sensitized B-cells);
- responses to booster doses, which may be planned for predefined subsets enrolled in studies or may be instituted when disease surveillance indicates a possible need;
- the possible increased risk of severe DFI in vaccine recipients (e.g. in some areas/regions it may be possible to collect data on hospitalization of vaccinees to capture severe dengue cases);
- surveillance of dengue in areas/regions where routine vaccination is introduced and, if possible, collection of sufficient data for a formal estimate of vaccine effectiveness.
There are several possible study designs and methods for estimating vaccine effectiveness and it is essential that expert advice is sought. In addition, it is likely that such studies would need to be performed in close liaison with public health authorities.

There may be no information on vaccine co-administration at the time of initial licensure. Sponsors and NRAs should consider the need to assess the interaction of any novel dengue vaccine with other vaccines that are likely to be co-administered. For instance, in countries where dengue vaccination will become part of the routine childhood immunization programme, the interaction with the other vaccines used in the programme needs to be studied. In addition, if licensure is sought in non-endemic areas with the intention of protecting travellers, it is advisable to study the possible interactions of a novel dengue vaccine with other vaccines for travellers. Interaction studies should assess safety and the immune response to all co-administered antigens.

Some or all post-licensure studies may be conducted as post-approval commitments made to an individual NRA. In this regard, both sponsors and NRAs that have approved a vaccine should communicate and cooperate to ensure that studies are well-designed to answer the questions posed and to avoid demands for numerous studies in individual countries that are likely to be too small to provide reliable results. Provisional plans for appropriate post-licensure studies should be submitted with the application dossier and these should be refined during the assessment by the NRA and as necessary after initial approval.

**Part D. Environmental risk assessment of dengue tetravalent vaccines (live, attenuated) derived by recombinant DNA technology**

**D.1 Introduction**

**D.1.1 Scope**

Some countries have legislation covering environmental and other concerns related to the use of live vaccines derived by recombinant DNA technology since they may be considered as GMOs. However, similar concerns may be raised by live vaccines derived by conventional methods.

This section of the Guidelines considers the environmental risk assessment (ERA) that may be performed during DENV vaccine development. The ERA assesses the risk to public health and the environment. It does not assess the risk to the intended recipient of the vaccine which is assessed through clinical studies of the vaccine. Nor does the ERA assess the risk to laboratory workers.

The environmental impact is not usually the responsibility of the NRA but of other agencies. Nonetheless the NRA should receive a copy of the ERA.
and of any associated decisions taken, both for information and to ensure that the appropriate procedures have been followed.

D.1.2 Principles and objectives

Live DENV vaccine in which the genome has been genetically modified by recombinant DNA technology is considered a GMO. The manufacture, use and transboundary shipping of such live recombinant vaccines for research or commercial use should, when applicable, comply with relevant legislation or regulations on GMOs in the producing and recipient countries. In some regulatory regimes, in order to comply with environmental regulations, an ERA should be undertaken if the live vaccine is being tested in a clinical trial or if it is placed on the market. It should be noted that the following guidance on the ERA of live recombinant DENV vaccines is not intended to replace existing GMO legislation that is already in place in certain countries.

Generally, the objective of an ERA is to identify and evaluate, on a case-by-case basis, the potential adverse effects (direct or indirect, immediate or delayed) of a GMO on public health and the environment. This means that a separate ERA should be performed for each different live recombinant dengue vaccine. “Direct effects” are primary effects on human health or on the environment which result from the GMO itself and which occur through a short causal chain of events. “Indirect effects” are effects that occur through a more extended causal chain of events, through mechanisms such as interactions with other organisms, transfer of genetic material, or changes in use or management. “Immediate effects” are observed during the period of the release of the GMO, whereas “delayed effects” are those effects which may not be observed during the period of release of the GMO but which become apparent as a direct or indirect effect either at a later stage or after termination of the release.

The ERA should be performed in a scientifically sound and transparent manner and should be based on available scientific and technical data. Important aspects to be addressed in an ERA include the characteristics of: (i) the parental organism, (ii) the recipient organism, (iii) viral vector characteristics, (iv) the donor sequence, (v) genetic modification, (vi) the intended use and (vii) the receiving environment. The data needed to evaluate the ERA do not have to derive solely from experiments performed by the applicant; data available in the scientific literature can also be used in the assessment. Regardless of the source, data should be both relevant and of an acceptable scientific quality. The ERA may be based on data from experiments previously performed for other purposes, such as product characterization tests and nonclinical safety and toxicity studies.

Ideally, the ERA is based on quantitative data and expressed in quantitative terms. However, much of the information that is available for an ERA may be qualitative since quantification is often difficult to accomplish and may not be
necessary to make a decision. The level of detail and information required in the ERA is also likely to vary according to the nature and the scale of the proposed release. Information requirements may differ between licensure and clinical development and according to whether studies will be carried out in a single country or multiple countries.

Uncertainty is inherent in the concept of risk. Therefore, it is important to identify and analyse areas of uncertainty in the risk assessment. Since there is no universally accepted approach for addressing uncertainty, risk management strategies may be considered. Precise data on the environmental fate of the live vaccine in early clinical trials will in most cases be insufficient or lacking. However, at the stage of market registration, the level of uncertainty is expected to be lower as gaps identified in available data should already have been addressed.

The need for risk management measures should be based on the estimated level of risk. If new information on the GMO becomes available, the ERA may need to be re-performed to determine whether the estimated level of risk has changed. This also holds true if the risks for the participating subjects have changed, as these aspects can be translated to other individuals. It should be noted that the ERA will not deal with medical benefit for the subject or scientific issues such as proof of principle.

D.2  **Procedure for environmental risk assessment**

Risk assessment involves identification of novel characteristics of the GMO that may have adverse effects (hazard), evaluation of the consequences of each potential adverse effect, estimation of the likelihood of adverse effects occurring, risk estimation, risk management and, in some methodologies, estimation of the overall risk to the environment. These processes should identify the potential adverse effects by comparing the properties of the GMO with those of non-modified organisms under the same conditions and in the same receiving environment. The principles and methodology of an ERA should be applicable irrespective of the geographical location of the intended environmental release of the GMO. However, the ERA should take into account the specificities associated with the mosquito vector being endemic or non-endemic in the region in which vaccine trials will be carried out, and/or where licensure is being requested. Depending on local regulatory requirements, the ERA may be undertaken by the applicant or by the competent local authority on the basis of data supplied. In all cases, the competent local authority should use the ERA as a basis for deciding whether any identified environmental risks are acceptable. Nevertheless, the decision on whether any identified risks are acceptable may vary from country to country. Several national and international documents address ERA issues (59–62).

The general process for undertaking an ERA is shown in Figure A2.1 as an example (60, 61).
Figure A2.1
Typical steps in an environmental risk assessment

| Step 1: Identification of characteristics which may cause adverse effects |
| Step 2: Evaluation of the potential consequences of each adverse effect, if it occurs |
| Step 3: Evaluation of the likelihood of the occurrence of each identified potential adverse effect |

| Step 4: Estimation of the risk posed by each identified characteristic of the GMO(s) |
| Step 5: Application of management strategies for risks from the deliberate release or marketing of GMO(s) |
| Step 6: Determination of the overall risk of the GMO(s) |

D.3 Special considerations for live recombinant dengue vaccines

The ERA of live recombinant DENV vaccines should be conducted according to the general principles described above, taking into consideration in particular the vector responsible for disease transmission. Aspects which could be developed include: the genetic stability of the live recombinant virus (including reversion and recombination), potential transmission of the vaccine virus among hosts by the vector, and the immune status of the population. These aspects are further outlined below.

D.3.1 Genetic stability

DENV vaccines currently under clinical evaluation are attenuated DENV strains, intertypic chimeric vaccines or DENV/yellow fever 17D vaccine chimeras. In the intertypic approach, the structural genes of an attenuated strain of DENV of a given serotype are replaced by the corresponding genes of a different DENV serotype. In the dengue/yellow fever chimeras, the prM/E structural genes of the dengue virus are cloned into the backbone of the yellow fever 17D vaccine, replacing the corresponding structural yellow fever 17D genes.

D.3.1.1 Reversion

After vaccination, there is potential for reversion of attenuated live dengue virus vaccines to a virulent form of the dengue virus, although this has not been seen in clinical trials so far. The potential reversion is based on the stability of the attenuating mutation(s), the number of attenuating mutations, and the nature of attenuating mutation. Attenuating mutations that are dependent on a single base change may be more susceptible to reversion than a mutation that is stabilized by multiple base substitutions. In addition, attenuating mutations that are derived by deletions of segments of RNA are generally more stable against reversion.
Changes in virus genotype have the potential to influence disease transmission, tropism of vector vaccine, virulence, and/or patterns of disease, resulting in a virus with a previously unknown combination of properties. However, the likelihood of such a reversion depends on the number of attenuation mutations present and the viral genes involved in the vaccine virus (63).

D.3.1.2 Recombination

Whether or not recombination takes place among flaviviruses is controversial. In theory, recombination between live DENV vaccines and wild-type flaviviruses could produce a virus with an altered phenotype, but there is currently no evidence to support this (64–70).

The potential for recombination within and between flaviviruses has been widely discussed and challenged in the past, both on the basis of existing literature (64–67, 69, 70) and also of data obtained in specific experiments. In particular, a “recombination trap” has recently been designed to allow the products of rare recombination events to be selected and amplified, in the case of West Nile encephalitis, tick-borne encephalitis and Japanese encephalitis viruses (69). Intergenomic but aberrant recombination was observed only in the case of Japanese encephalitis virus, and not for West Nile or tick-borne encephalitis viruses. Moreover, its frequency appeared to be very low and generated viruses with impaired growth properties.

While their likelihood of appearance is very low, as stated above, the potential adverse effects of recombined DENVs should be evaluated in the ERA. In this respect, “worst-case” scenarios for chimeras have been constructed to address that risk (65, 66, 70).

These different studies showed that such recombinants constructed artificially from a wild-type flavivirus and a chimeric vaccine (70), or from two wild-type viruses, such as highly virulent yellow fever Asibi virus and wild-type DEN-4 virus (66), were highly attenuated compared to their parental viruses. Attenuation was shown in culture in vitro, in mosquito vectors and in susceptible animal models, including monkeys. These data provide experimental evidence that the potential of recombinants, should they ever emerge, to cause disease or spread would probably be very low. Dual infection laboratory studies between vaccine and wild-type strains are not recommended because the predictive clinical value of such studies would be low.

D.3.2 Vector transmission

The presence of the DENV vectors such as *Ae. aegypti* and *Ae. albopictus* play a key role in the transmission of flaviviruses and potentially of live DENV vaccines from the vaccinated subject to other individuals. Dengue does not spread directly from person to person, except via blood transfusion in very rare instances where a donor was dengue viraemic. Transmission of the dengue
vaccine in regions where the vector is absent is therefore highly unlikely. Dengue is currently restricted to the tropical and a few subtropical regions. Due to climate change there is the possibility of a geographical shift in mosquito populations which could conceivably lead to the spread of dengue to areas that are currently non-endemic.

Recombination between live DENV vaccines and wild-type flaviviruses could theoretically occur in a vaccinee (see above) and possibly also within an infected mosquito, although neither has been reported. A recombined DENV could potentially, for instance in combination with climate change, use new vectors for transmission, leading to previously unknown transmission characteristics. Therefore, the presence of a relevant mosquito vector and a “dengue favourable climate” in the vaccination region should be taken into account in the ERA of live DENV vaccines.

To assess the likelihood of effective transmission of the vaccine from a vaccinated individual, two parameters should be taken into consideration: the level of viraemia in the vaccinated hosts, and the ability of the mosquito vectors to transmit the live DENV vaccine to new hosts. The blood titre required for effective transmission of dengue virus from human to mosquito via the bite has been studied in a laboratory setting. The typical level and duration of vaccine viraemia in inoculated volunteer subjects is also known for all live vaccines currently in the clinical phase of development. These data show that peak titres of vaccine viraemia are several orders of magnitude below those needed to infect a mosquito (71). In addition, the ability of the live vaccine viruses to replicate in mosquitoes and then to escape the midgut in order to render the mosquito infectious for humans by entering the salivary glands is also very impaired compared to wild-type dengue (18, 19, 26, 30, 54, 72, 73). Thus it is highly unlikely that vaccinated subjects could ever spread vaccine virus via mosquito transmission.

The outcome of the ERA for clinical trials in regions where the vector is absent is obviously that the environmental risk is negligibly small. The mosquito vector is not present and therefore the vaccine, or theoretical de novo recombinant viruses, cannot be transmitted to other people. However, in endemic areas, NRAs should decide whether or not to perform an ERA.

D.3.3 Immune status

Live DENV vaccines are able to replicate in vaccinated persons. The immune status against the vaccine antigens, the viral vectors and/or cross-reacting flaviviruses in the vaccinee may be a confounding factor in the assessment of the environmental risk of a live DENV vaccine. In general, the presence of pre-existing immunity due to earlier exposure to DENVs will reduce the extent and duration of vaccine virus replication and dissemination within a vaccinee. The potential for transmission of the vaccine is therefore considered to be greater in naive or immunocompromised individuals.
An unvaccinated population with no pre-existing immunity will respond differently upon exposure to the vaccine compared to a population in which dengue is endemic. The immune status should therefore be taken into account in the ERA as it can influence both the environmental impact of the vaccines and the potential occurrence of adverse effects in contacts of the vaccinees. There is a theoretical potential for pre-existing heterotypic antibody to cause higher levels of vaccine virus. Enhanced illness in vaccine recipients who have pre-existing DENV antibody (antibody-dependent enhancement) has not been observed in clinical trials of live, attenuated dengue vaccines to date and was not observed in a clinical trial of live, attenuated dengue vaccines designed to address this possibility (74, 75).

Part E. Guidelines for NRAs

E.1 General
The general recommendations for NRAs and NCLs provided in the Guidelines for national authorities on quality assurance for biological products (76) should apply. In addition, the general recommendations for NRAs and NCLs provided in the Guidelines for independent lot release of vaccines by regulatory authorities (77) should be followed. These Guidelines specify that no new biological substance should be released until consistency of manufacturing and quality, as demonstrated by a consistent release of batches, has been established. The detailed production and control procedures, and any significant changes in them, should be discussed with and approved by the NRA. The NRA should obtain the working reference from manufacturers to establish a national working Reference Preparation until an international Reference Reagent is available.

E.2 Release and certification
A vaccine lot should be released only if it fulfils 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 NRA in support of a request for release of vaccine for use.

A statement signed by the appropriate official of the NRA should be provided if requested by a manufacturing establishment, and should certify whether or not the lot of vaccine in question meets all national requirements, as well as Part A of these 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 antigen 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 dengue virus vaccines between countries.

Authors

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The fourth draft was prepared by Dr A. Barrett, University of Texas Medical Branch, Galveston, TX, USA; Dr D. Bleijs, National Institute for Public Health and the Environment, Bilthoven, the Netherlands; Dr P. Minor, National Institute of Biological Standards and Control, Potters Bar, England; Dr M. Powell, Medicines and Healthcare Products Regulatory Agency, London, England; Dr J. Shin, World Health Organization, Geneva, Switzerland; and Dr D. Trent, University of Texas Medical Branch, Galveston, TX, USA, taking into account suggestions for modification and comments by the participants in the informal consultation held on 11–12 April 2011 in Geneva, Switzerland, attended by: Dr B. Barrere, Sanofi Pasteur, Marcy l’Étoile, France; Dr A. Barrett, University of Texas Medical Branch, Galveston, TX, USA; Dr D. Bleijs, National Institute for Public Health and the Environment, Bilthoven, the Netherlands; Dr A. Chawla, Greater Noida, Uttar Pradesh, India; Dr K. Dobbelkaere, GlaxoSmithKline Biologicals, Rixensart, Belgium; Dr M. Dornbusch, Office of the Gene Technology Regulator, Department of Health and Aging, Canberra, Australia; Dr A. Durbin, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, USA; Dr K. Eckels, Walter Reed Army Institute of Research, Silver Spring, MD, USA; Dr R. Edelman, University of Maryland School of Medicine, Baltimore, MD, USA; Mr M. Galves, National Agency of Health Surveillance, Brasilia-DF, Brazil; Dr E. Griffiths, Biologics and Genetic Therapies Directorate, Health Canada, Ottawa, Canada; Dr J. Hombach, World Health Organization, Geneva, Switzerland; Dr I. Knezevic, World Health Organization, Geneva, Switzerland; Dr L. Mallet, Sanofi Pasteur, Toronto, Canada; Dr L. Markoff, Center for Biologics Evaluation and Research,
Food and Drug Administration, Bethesda, MD, USA; Dr P. Minor, National Institute of Biological Standards and Control, Potters Bar, England (Chair); Dr L. Morgan, Sanofi Pasteur, Marcy l’Étoile, France; Dr J. Korimbocus, Agence Française de Sécurité sanitaire de Produits de Santé (French Agency for Safety of Health Products), Lyons, France; Dr M. Powell, Medicines and Healthcare Products Regulatory Agency, London, England; Dr A. Precioso, Butantan, Sao Paulo, Brazil; Dr V. Quivy, GlaxoSmithKline Biologicals, Wavre, Belgium; Dr J. Roehrig, Centers for Disease Control and Prevention, Fort Collins, CO, USA; Dr J. Schmitz, World Health Organization, Geneva, Switzerland; Mrs P. Thanaphollert, Food and Drug Administration, Ministry of Public Health, Nonthaburi, Thailand; Dr D. Trent, University of Texas Medical Branch, Galveston, TX, USA; Dr J-W. van der Laan, National Institute for Public Health and the Environment, Bilthoven, the Netherlands; and Dr S. Viviani, Sanofi Pasteur, Marcy l’Étoile, France. This fourth draft was posted on the WHO web site with a call for public comments for one month from 22 May to 23 June 2011.

The fifth draft was prepared by the same members of the drafting group that prepared the fourth, and was submitted to the Expert Committee on Biological Standardization for consideration. This draft was posted on the WHO web site with a call for public comments for two months from 21 July to 23 September 2011.

The document was further modified and then adopted by the WHO Expert Committee on Biological Standardization in October 2011.

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References


Appendix 1

Summary protocol for manufacturing and control of dengue tetravalent vaccine (live, attenuated)

The following protocol is intended for guidance, and indicates the information that should be provided as a minimum by the manufacturer to the NRA. Information and tests may be added or omitted as necessary to be in line with the marketing authorization approved by the NRA. It is therefore 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 on a particular product should be given in the protocol submitted. The section concerning the final product should 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 should also be accompanied by a lot release certificate from the NRA of the country in which the vaccine was produced and/or released stating that the product meets national requirements as well as Part A of the Guidelines of this document published by WHO.

1. Summary information on finished product (final vaccine lot)

International name: ________________________________
Commercial name: ________________________________
Product licence (marketing authorization) number: ________________
Country: ________________________________
Name and address of manufacturer: ________________________________
Name and address of product licence holder if different: ________________________________
Virus strains: ________________________________
Origin and short history: ________________________________
Batch number(s): ________________________________
Finished product (final lot): ________________________________
Final bulk: ________________________________
Type of container: ________________________________
Number of filled containers in this final lot: ________________________________
Number of doses per container: ________________________________
Composition (antigen concentration)/ volume of single human dose: ________________________________
Target group: ________________________________
2. Summary information on manufacture

Batch number of each monovalent bulk: ________________________________
Site of manufacture of each monovalent bulk: ________________________
Date of manufacture of each monovalent bulk: ________________________
Batch number of final bulk: ________________________________
Site of manufacture of final bulk: ________________________
Date of manufacture of final bulk: ________________________
Date of manufacture (filling or lyophilizing) of finished product (final vaccine lot): ________________________
Date on which last determination of virus concentration was started: ________________________
Shelf-life approved (months): __________________________
Storage conditions: ________________________________
Volume of single dose: ________________________________
Prescribed virus concentration per human dose:
  Serotype 1: ________________________________
  Serotype 2: ________________________________
  Serotype 3: ________________________________
  Serotype 4: ________________________________

A genealogy of the lot numbers of all vaccine components used in the formulation of the final product will be informative.

The following sections are intended to report the results of the tests performed during production of the vaccine.

3. Control of source materials

3.1 Cell cultures

3.1.1 General information on cell banking system

Information and results of characterization tests on the cell banking system from cell seed (if applicable), master cell bank, working cell bank, end-of-production cells or extended cell bank should be provided according to WHO's Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks.

Name and identification of cell substrate: ________________________________
Origin and short history (attach a flowchart if necessary): ________________________________
Lot number and date of preparation for each bank: ____________________________
Date each bank was established: __________________________________________
Date of approval by the NRA: _____________________________________________
Total number of ampoules stored for each bank: _____________________________
Passage/population doubling level of each bank: _____________________________
Maximum passage/population doubling level approved for each bank: ____________
Storage conditions: ______________________________________________________
Date of approval of protocols indicating compliance with the requirements of the relevant monographs and with the marketing authorization: ________________

3.1.2 Characterization tests on cell seed (if applicable), master cell bank, working cell bank, end-of-production cells, or extended cell banks

A summary table for characterization tests on each bank should be provided.

Characterization tests performed on each bank
   Methods: __________________________________________________________________
   Specifications: __________________________________________________________________
   Date tested: ___________________________________________________________________
   Results: ______________________________________________________________________

3.1.3 Cell culture medium

Serum used in cell culture medium
   Animal origin of serum: ______________________________________________________
   Batch number: __________________________________________________________________
   Vendor: _________________________________________________________________________
   Country of origin: __________________________________________________________________
   Certificate of TSE-free: __________________________________________________________________
   Tests performed on serum
      Methods: ______________________________________________________________________
      Specifications: ____________________________________________________________________
      Date of test: _____________________________________________________________________
      Results: _______________________________________________________________________

Trypsin used for preparation of cell cultures
   Animal origin of trypsin: _________________________________________________________
   Batch number: ___________________________________________________________________
   Vendor: _________________________________________________________________________
   Country of origin: __________________________________________________________________
   Certificate of TSE-free: __________________________________________________________________
Tests performed on trypsin

Methods: 
Specifications: 
Date of test: 
Results: 

Antibiotics
Nature and concentration of antibiotics or selecting agent(s) used in production cell culture maintenance medium: 

Other source material
Identification and source of starting materials used in preparing production cells, including excipients and preservatives (particularly any materials of human or animal origin, e.g. albumin, serum): 

3.2 Virus seeds
Vaccine virus strain(s) and serotype(s): 
Substrate used for preparing seed lots: 
Origin and short history: 
Authority that approved virus strain(s): 
Date approved: 

3.2.1 Information on seed lot preparation
Virus master seed
Source of virus master seed lot: 
Virus master seed lot number: 
Name and address of manufacturer: 
Passage level: 
Date of inoculation: 
Date of harvest: 
Number of containers: 
Conditions of storage: 
Date of establishment: 
Maximum passage level approved for virus master seed: 
Date approved by the NRA: 

Virus working seed
Virus working seed lot number: 
Name and address of manufacturer: 
Passage level from virus master seed lot: __________________________
Date of inoculation: __________________________
Date of harvest: __________________________
Number of containers: __________________________
Conditions of storage: __________________________
Date of establishment: __________________________
Date approved by the NRA: __________________________

3.2.2 Tests on virus seeds

Identity test
Method: __________________________
Specification: __________________________
Lot number of reference reagents: __________________________
Dates of test (start, end): __________________________
Result: __________________________

Genetic/phenotypic characterizations
Method: __________________________
Reference reagents: __________________________
Specification: __________________________
Dates of test (start, end): __________________________
Result: __________________________

Tests for bacteria and fungi
Method: __________________________
Specification: __________________________
Media: __________________________
Number of containers tested: __________________________
Volume of inoculum per container: __________________________
Volume of medium per container: __________________________
Temperatures of incubation: __________________________
Dates of test (start, end): __________________________
Result: __________________________

Test for mycoplasmas
Method: __________________________
Specification: __________________________
Media: __________________________
Volume tested: __________________________
Temperature of incubation: __________________________
Positive controls: __________________________
Dates of test (start, end): __________________________________________
Result: _______________________________________________________

**Test for mycobacteria**
Method: _______________________________________________________
Specification: _________________________________________________
Media: ________________________________________________________
Volume tested: ________________________________________________
Temperature of incubation: ____________________________________
Dates of test (start, end): _______________________________________
Result: _______________________________________________________

**Adventitious agents**
Volume of virus seed samples for neutralization and testing: _______
Batch number(s) of antisera/antiserum used for neutralization of virus seeds: _________________________

**Test in tissue cultures for adventitious agents**
**Test in monkey cells**
Type of monkey cells: __________________________________________
Quantity of neutralized sample inoculated: _________________________
Incubation conditions: _________________________________________
Method: _______________________________________________________
Specification: _________________________________________________
Dates of test (start, end): _______________________________________
Ratio of cultures viable at end of test: _____________________________
Result: _______________________________________________________

**Test in human cells**
Type of human cells: __________________________________________
Quantity of neutralized sample inoculated: _________________________
Incubation conditions: _________________________________________
Method: _______________________________________________________
Specification: _________________________________________________
Dates of test (start, end): _______________________________________
Ratio of cultures viable at end of test: _____________________________
Result: _______________________________________________________

**Other cell types**
Type of cells: _________________________________________________
Quantity of neutralized sample inoculated: _____________________
Incubation conditions: ________________________________
Method: ________________________________
Specification: ________________________________
Dates of test (start, end): ________________________________
Ratio of cultures viable at end of test: _____________________
Result: ________________________________

Test in animals for adventitious agents
Method: ________________________________
Specification: ________________________________
Dates of test (start, end): ________________________________
Result: ________________________________

Test by molecular methods for adventitious agents
Method: ________________________________
Specification: ________________________________
Dates of test (start, end): ________________________________
Result: ________________________________

Tests in nonhuman primates (either master or working seed lot) for neurovirulence
For details, please see Recommendations for yellow fever vaccine (51)
Method: ________________________________
Specification: ________________________________
Dates of test (start, end): ________________________________
Result: ________________________________

Tests in suckling mice (either master or working seed lot, where necessary) for neurovirulence
(Detailed protocol should be developed)
Method: ________________________________
Specification: ________________________________
Dates of test (start, end): ________________________________
Result: ________________________________

Virus titration for infectivity
Method: ________________________________
Specification: ________________________________
Dates of test (start, end): ________________________________
Result: ________________________________
4. Control of vaccine production

4.1 Control of production cell cultures

4.1.1 Information on preparation

Lot number of master cell bank: ________________________________
Lot number of working cell bank: ________________________________
Date of thawing ampoule of working cell bank: __________________
Passage number of production cells: _____________________________
Date of preparation of control cell cultures: ______________________
Result of microscopic examination: ______________________________

4.1.2 Tests on control cell cultures

Amount or ratio of control cultures to production cell cultures: __________________________
Incubation conditions: __________________________________________
Period of observation of cultures: _________________________________
Dates started/ended: _____________________________________________
Ratio of cultures discarded and reason: _____________________________
Results of observation: __________________________________________
Date supernatant fluid collected: _________________________________

Test for haemadsorbing viruses

Quantity of cells tested: ________________________________
Method: _________________________________________________
Specification: ____________________________________________
Dates of test (start, end): _________________________________
Result: __________________________________________________

Test for adventitious agents on supernatant culture fluids

Test in monkey cells

Type of monkey cells: ________________________________
Quantity of pooled sample inoculated: __________________________
Incubation conditions: ______________________________________
Method: _________________________________________________
Specification: ____________________________________________
Dates of test (start, end): _________________________________
Ratio of cultures viable at end of test: __________________________
Result: __________________________________________________

Test in human cells

Type of human cells: ________________________________
Quantity of pooled sample inoculated: __________________________
Incubation conditions: ______________________________
Method: ______________________________
Specification: ______________________________
Dates of test (start, end): ______________________________
Ratio of cultures viable at end of test: ______________________________
Result: ______________________________

Other cell types
Type of cells: ______________________________
Quantity of pooled sample inoculated: ______________________________
Incubation conditions: ______________________________
Method: ______________________________
Specification: ______________________________
Dates of test (start, end): ______________________________
Ratio of cultures viable at end of test: ______________________________
Result: ______________________________

Identity test
Method: ______________________________
Specification: ______________________________
Dates of test (start, end): ______________________________
Result: ______________________________

4.1.3 Cells used for vaccine production
Observation of cells used for production
Specification: ______________________________
Date: ______________________________
Result: ______________________________

4.2 Monovalent virus harvest pools
4.2.1 Information on manufacture

*Information on each monovalent virus harvest pool should be provided separately.*
Batch number(s): ______________________________
Date of inoculation: ______________________________
Date of harvesting: ______________________________
Lot number of virus master seed lot: ______________________________
Lot number of virus working seed lot: ______________________________
Passage level from virus working seed lot: ______________________________
Methods, date of purification if relevant: ______________________________
Volume(s), storage temperature, storage time and approved storage period: ______________________________
4.2.2 Tests on monovalent virus harvest pools

Identity
Method: ____________________________________________
Specification: ______________________________________
Lot number of reference reagents: _______________________
Specification: ______________________________________
Date of test: ________________________________________
Result: ____________________________________________

Test for bacteria and fungi
Method: ____________________________________________
Specification: ______________________________________
Media: _____________________________________________
Number of containers tested: _________________________
Volume of inoculum per container: ____________________
Volume of medium per container: _____________________
Temperatures of incubation: ___________________________
Dates of test (start, end): _____________________________
Result: ____________________________________________

Test for mycoplasma
Method: ____________________________________________
Specification: ______________________________________
Media: _____________________________________________
Volume tested: _____________________________________
Temperature of incubation: ___________________________
Positive controls: ___________________________________
Dates of test (start, end): _____________________________
Result: ____________________________________________

Test for mycobacteria
Method: ____________________________________________
Specification: ______________________________________
Media: _____________________________________________
Volume tested: _____________________________________
Temperature of incubation: ___________________________
Dates of test (start, end): _____________________________
Result: ____________________________________________

Test for adventitious agents
Test in monkey cells
Type of monkey cells: _______________________________
Quantity of neutralized sample inoculated: ______________________
Incubation conditions: ________________________________
Method: _________________________________________
Specification: ______________________________________
Dates of test (start, end): _____________________________
Ratio of cultures viable at end of test: ____________________
Result: ____________________________________________

Test in human cells
Type of human cells: _________________________________
Quantity of neutralized sample inoculated: ______________________
Incubation conditions: ________________________________
Method: _________________________________________
Specification: ______________________________________
Dates of test (start, end): _____________________________
Ratio of cultures viable at end of test: ____________________
Result: ____________________________________________

Other cell types
Type of cells: _________________________________
Quantity of neutralized sample inoculated: ______________________
Incubation conditions: ________________________________
Method: _________________________________________
Specification: ______________________________________
Dates of test (start, end): _____________________________
Ratio of cultures viable at end of test: ____________________
Result: ____________________________________________

Virus titration for infectivity
Method: _________________________________________
Specification: ______________________________________
Dates of test (start, end): _____________________________
Result: ____________________________________________

Test for host cell proteins
Method: _________________________________________
Specification: ________________
Date of test: ______________________
Result: _________________________________________

Test for residual cellular DNA
Method: _________________________________________
4.3. **Final tetravalent vaccine bulk**

### 4.3.1 Information on manufacture

- **Batch number(s):**
- **Date of formulation:**
- **Total volume of final bulk formulated:**
- **Monovalent virus pools used for formulation:**
- **Serotype/lot number/volume added/virus concentration:**
- **Name and concentration of added substances (e.g. diluent, stabilizer if relevant):**
- **Volume(s), storage temperature, storage time and approved storage period:**

### 4.3.2 Tests on final tetravalent bulk lot

#### Residual animal serum protein

- **Method:**
- **Specification:**
- **Dates of test (start, end):**
- **Result:**

#### Test for bacteria and fungi

- **Method:**
- **Specification:**
- **Media:**
- **Number of containers tested:**
- **Volume of inoculum per container:**
- **Volume of medium per container:**
- **Temperatures of incubation:**
- **Dates of test (start, end):**
- **Result:**
5. Filling and containers

Lot number: ________________________________
Date of filling: ________________________________
Type of container: ________________________________
Volume of final bulk filled: ________________________________
Filling volume per container: ________________________________
Number of containers filled (gross): ________________________________
Date of lyophilization: ________________________________
Number of containers rejected during inspection: ________________________________
Number of containers sampled: ________________________________
Total number of containers (net): ________________________________
Maximum period of storage approved: ________________________________
Storage temperature and period: ________________________________

6. Control tests on final vaccine lot

6.1 Tests on vaccine lot

Inspection of final containers

Appearance: ________________________________
Specification: ________________________________
Date of test: ________________________________
Results: ________________________________
Before reconstitution: ________________________________
After reconstitution: ________________________________
Diluent used: ________________________________
Lot number of diluent used: ________________________________

Test for pH

Method: ________________________________
Specification: ________________________________
Date of test: ________________________________
Result: ________________________________

Identity test (each serotype)

Method: ________________________________
Specification: ________________________________
Date of test: ________________________________
Result: ________________________________

Test for bacteria and fungi

Method: ________________________________
Specification: ________________________________
Media: 
Volume tested: 
Temperatures of incubation: 
Dates of test (start, end): 
Result: 

Test for potency (each serotype)
Method: 
Batch number of reference vaccine and assigned potency: 
Specification: 
Dates of test (start, end): 
Result for each serotype: 

Thermal stability (each serotype)
Method: 
Specification: 
Dates of test (start, end): 
Result for each serotype: 

General safety (unless deletion authorized)
Tests in mice
Date of inoculation: 
Number of animals tested: 
Volume and route of injection: 
Observation period: 
Specification: 
Results (give details of deaths): 

Tests in guinea-pigs
Date of inoculation: 
Number of animals tested: 
Volume and route of injection: 
Observation period: 
Specification: 
Results (give details of deaths): 

Residual moisture
Method: 
Specification: 
Date: 
Result: 
Residual antibiotics if applicable

Method: ________________________________
Specification: ________________________________
Date: ________________________________
Result: ________________________________

6.2 Diluent

Name and composition of diluent: ________________________________
Lot number: ________________________________
Date of filling: ________________________________
Type of diluent container: ________________________________
Filling volume per container: ________________________________
Maximum period of storage approved: ________________________________
Storage temperature and period: ________________________________

7. Certification by the manufacturer

Name of head of production (typed) ________________________________

Certification by the 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 dengue vaccine, whose number appears on the label of the final containers, meets all national requirements and satisfies Part A1 of the WHO Guidelines on the quality, safety and efficacy of dengue tetravalent vaccines (live, attenuated) (2013)2 (if applicable)

Name (typed) ________________________________
Signature ________________________________
Date ________________________________

8. Certification by the NRA

If the vaccine is to be exported, attach a certificate from the NRA (as shown in Appendix 2), a label from a final container, and an instruction leaflet for users.

---

1 With the exception of provisions on distribution and shipping, which the NRA may not be in a position to assess.
Appendix 2

Model certificate for the release of dengue tetravalent vaccine (live, attenuated) by NRAs

This certificate is to be provided by the NRA of the country where the vaccine has been manufactured, on request by the manufacturer.

Lot release certificate

Certificate no. ________________

The following lot(s) of dengue vaccine produced by ________________ in ________________, whose numbers appear on the labels of the final containers, complies with the relevant specification in the marketing authorization and provisions for the release of biological products and Part A of the WHO Guidelines on the quality, safety and efficacy of dengue tetravalent vaccines (live, attenuated) (2013) and comply with WHO good manufacturing practices: main principles for pharmaceutical products; Good manufacturing practices for biological products; and Guidelines for independent lot release of vaccines by regulatory authorities.

The release decision is based on ________________

The certificate may include the following information:

- name and address of manufacturer;
- site(s) of manufacturing;
- trade name and common name of product;

---

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 NRA.
4 With the exception of provisions on distribution and shipping, which the NRA may not be in a position to assess.
9 Evaluation of summary protocol, independent laboratory testing, and/or specific procedures laid down in defined document etc., as appropriate.
- marketing authorization number;
- lot number(s) (including sub-lot numbers, packaging lot numbers if necessary);
- type of container;
- number of doses per container;
- number of containers/lot size;
- date of start of period of validity (e.g. manufacturing date) and/or expiry date;
- storage condition;
- signature and function of the authorized person and authorized agent to issue the certificate;
- date of issue of certificate;
- certificate number.

The Director of the NRA (or other authority as appropriate):

Name (typed) ____________________________
Signature _______________________________
Date _________________________________
Annex 3

Recommendations to assure the quality, safety and efficacy of BCG vaccines

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

The last revision of the Requirements for dried bacille Calmette–Guérin (BCG) vaccine for human use was in 1985, and an amendment which updated the section on the expiry date was published in 1988 (1, 2). Recent WHO consultation meetings (3–6) have addressed issues concerning the improvement of vaccine characterization and quality control assays of BCG vaccine to reflect current state-of-the-art technology. In addition, a recommendation to replace the International Reference Preparation for BCG vaccine by substrain-specific Reference Reagents evaluated by collaborative studies has been proposed. This document provides: recommendations for the production and control of BCG vaccines (Part A); guidelines for nonclinical evaluation (Part B); guidelines for the content of the clinical development programme applicable to BCG vaccines (Part C); and recommendations for NRAs (Part D). The guidelines for nonclinical evaluation apply to classic BCG vaccine products that are still in need of such evaluation, including newly manufactured products requiring clinical trial studies or those produced following changes in the manufacturing process. The clinical part of this document aims to provide a basis for assessment of efficacy and safety of BCG vaccines in pre-licensing clinical trials as well as in post-marketing surveillance, monitoring consistency of production and clinical testing of new classic BCG vaccine products. If important changes have been introduced to an authorized production process, the need for preclinical and clinical testing should be considered on a case-by-case basis in consultation with the NRA(s) concerned.

General considerations

Tuberculosis (TB) was declared a global emergency by WHO in 1993, and Mycobacterium tuberculosis (M. tuberculosis) is now considered to be responsible for more adult deaths than any other pathogen. Vaccination with BCG still remains the standard for TB prevention in most countries because of its efficacy in preventing life-threatening forms of TB in infants and young children. It is inexpensive and usually requires only one administration in either newborns or adolescents (7, 8). As there is currently no suitable alternative, BCG will remain in use for the foreseeable future and may continue to be used as a prime vaccine in a prime-boost immunization schedule in conjunction with new TB vaccines (4).

BCG vaccine contains a live, attenuated strain of M. bovis that was originally isolated from cattle with tuberculosis and cultured for a period of 13 years and a total of 231 passages (7). The BCG vaccine was first used to immunize humans in 1921. Following its introduction into the WHO Expanded Programme on Immunization (EPI) in 1974, the vaccine soon reached global coverage rates exceeding 80% in countries endemic for TB (9).
Over the years, different BCG vaccine seed strains have evolved from the original vaccine strain for production. A number of BCG vaccine strains that are used worldwide differ in terms of their genetic and phenotypic properties, and their reactogenicity and immunogenicity profile when given to infants and children. With this background of a diversity of substrains, manufacturing processes, immunization schedules and levels of exposure to environmental mycobacteria and virulent *M. tuberculosis* infection, different levels of protective efficacy of BCG vaccines in adult populations have been reported (10). However, the data are insufficient to make recommendations on whether one strain should be preferred over the other (11). The United Nations agencies are the largest supplier of BCG vaccines, distributing more than 120 million doses each year to more than 100 countries. Worldwide, the most commonly used vaccine strains are currently Danish 1331, Tokyo 172-1 and Russian BCG-I because they are supplied by the United Nations Children’s Fund (UNICEF) which purchases the vaccines through a published prequalification process which determines their eligibility for use in national immunization programmes (12).

There has been particular concern over the safety of BCG vaccination in subjects infected with the human immunodeficiency virus (HIV) (8). WHO previously recommended that in countries with a high burden of TB, a single administration of BCG vaccine should be given to all healthy infants as soon as possible after birth, unless the child presented a symptomatic HIV infection (9). However, recent evidence shows that children who were HIV-infected when vaccinated with BCG at birth, and who later developed acquired immunodeficiency syndrome (AIDS), were at increased risk of developing disseminated BCG disease. Among these children, the benefits of potential prevention of severe TB are outweighed by the risks associated with the use of BCG vaccine; thus the use of BCG vaccines at birth in relation to HIV-infected infants should follow the recommendations of the Global Advisory Committee on Vaccine Safety (GACVS) (13, 14).

**Special considerations**

The formulation of international requirements for freeze-dried BCG vaccine is complicated by the following: (a) a number of different substrains derived from the original strain of BCG are used in vaccine manufacture; (b) a number of different manufacturing and testing procedures are employed; (c) it is difficult to identify a link between significant differences in vitro and in vivo between different BCG vaccine strains and any possible differences in protective efficacy against TB in humans; (d) vaccines are produced with different total bacterial content and numbers of culturable particles; and (e) vaccines intended for administration by different routes are prepared. Therefore, the following considerations should be borne in mind regarding the scope of these recommendations, BCG vaccine strains, and potency-related tests.
Scope of the Recommendations

These revised Recommendations refer to freeze-dried BCG vaccines prepared from substrains derived from original BCG for use in the prevention of TB. Where BCG vaccine is issued in liquid form, the application of these Recommendations is entirely the responsibility of the NRA. In that case, only the relevant parts of this document apply since the limited stability of liquid BCG limits the possibility of completing the full recommended control test schedule. Although many of the principles expressed in this document (e.g. manufacturing, quality control) are expected to apply also to new recombinant BCG and other live, attenuated mycobacterial vaccines modified by molecular biology techniques, these novel vaccines are outside the scope of these Recommendations. The same pertains to the use of BCG for immunotherapy (e.g. treatment of bladder cancer). However, applicability of issues on nonclinical and clinical evaluations should be considered on a case-by-case basis. These Recommendations have been formulated primarily to cover vaccines intended for intradermal and percutaneous administration. Although WHO recommends intradermal administration of the vaccine, preferably in the deltoid region of the arm using syringe and needle, other administration methods such as percutaneous application by the multiple puncture technique are practised in some countries (9, 15–17).

BCG vaccine strains

The original BCG vaccine strain was formerly distributed by the Pasteur Institute of Paris and subcultured in different countries using different culture conditions that were not standardized. Over the years, more than 14 substrains of BCG have evolved and have been used as BCG vaccine strains in different parts of the world (see Appendix 1). Recently, the various substrains have been studied by comparative genomics (18, 19). BCG vaccine strains were thus divided into the “early” strains, in which the original characteristics of “authentic Pasteur” were conserved with fewer deletions, insertions and mutations in the genome of the bacilli than the “late” strains. “Early” strains are represented by BCG Russia BCG-I, BCG Moreau-RJ, BCG Tokyo 172-1, BCG Sweden, and BCG Birkhaug; and the “late” strains include BCG Pasteur 1173P2, BCG Danish 1331, BCG Glaxo (Copenhagen 1077) and BCG Prague. The genomic sequences of BCG Pasteur 1173P2 as a “late” strain, and BCG Tokyo 172-1 and BCG Moreau as “early” strains were determined (18–20). There is insufficient direct evidence to suggest that various BCG substrains differ significantly in their efficacy to protect against TB in humans. However, evidence from animal and human studies indicates differences in the immune responses induced by different BCG vaccine strains (12, 21). Although the “early” strains may confer better protection against
TB in some animal studies (18, 22), commonly administered BCG vaccine strains including both evolutionary “early” and “late” strains induce comparable protective immunity against TB (23).

Only master seed lots that have been shown to be acceptable by laboratory and clinical tests on batches derived from them should be used for the production of working seed lots and/or final product. A suitable seed lot of BCG should yield vaccines that give protection in experimental animals, produce a relatively high level of immunological responses to *M. tuberculosis* antigens including tuberculin sensitivity in humans, and have an acceptably low frequency of adverse reactions (see section A.3.1).

Some manufacturers of freeze-dried BCG vaccine have modified their master seed lot strain to make it more suitable for their particular production procedure. The seed lots prepared in this way may not retain the same immunogenic properties, and should be used only with the approval of the NRA.

In practice, a product prepared from BCG seed lots may generally be investigated in humans only for the properties of producing tuberculin sensitivity and vaccination lesions. The former should be measured by the distribution of tuberculin reactions according to size in persons vaccinated with a given dose of BCG vaccine. A low dose of tuberculin should be employed (e.g. equivalent to 5 IU of the First WHO International Standard for purified protein derivative (PPD) of *M. tuberculosis*, or 2 tuberculin units (TU) of a batch of PPD RT23 with Tween 80).

Currently three substrain-specific Reference Reagents for BCG vaccines are available: BCG Danish 1331, Tokyo 172-1 and Russian BCG-I.

**Potency-related tests**

There is some evidence that BCG seed lots that have been shown to produce vaccines with protective potency in laboratory animals and tuberculin sensitivity in humans will give effective protection against TB in humans. It should be noted that tuberculin sensitivity is a marker for cell-mediated immune responses to mycobacteria and not a direct indicator of protective immunity. A number of alternative laboratory tests have been developed primarily for research purposes but, to date, none have been proved reliable indicators of protective immune conversion following administration of different vaccines.

Studies in animals should include protection tests, tests of vaccination lesions, and tests for tuberculin conversion. Immunizing efficacy should be measured in terms of degree of protection afforded to the test animals against a challenge with virulent *M. tuberculosis*. Sensitizing efficacy should be measured by the average dose of vaccine that will convert a negative tuberculin reaction in guinea-pigs to a positive one, as well as by the reaction time during which
the conversion takes place. In these animal tests, the inclusion for comparative purposes of an in-house reference BCG vaccine prepared from a seed lot known to be effective in animals and humans is recommended.

Currently there is no biomarker which directly correlates to clinical efficacy of BCG vaccine. These Recommendations are intended to be used for ensuring the manufacture of consistent lots. This means that new lots should not significantly differ from those that have already been shown to be safe and effective in humans.

At present, for batch control purposes, much reliance is placed on tests for the estimation of the total bacterial content and for the number of culturable particles. It is not possible to specify single requirements for the total bacterial content and for the number of culturable particles for all vaccines (24), since different substrains and methods of manufacture may yield different specifications for these parameters. For example, although the number of culturable bacteria in a single human dose may differ for different vaccines, these vaccines may show satisfactory properties as regards their ability to induce adequate sensitivity to tuberculin and their safety in humans. It is therefore essential that clinical studies for dose optimization in humans be carried out to estimate suitable total bacterial contents and the number of culturable particles for a particular manufacturer's product. For a particular vaccine, the difference between the lower and upper specification for the number of culturable particles should not be larger than fourfold. In addition, it is necessary to perform animal experiments that give an indication of the safety and efficacy of the vaccines to the satisfaction of the NRA.

**Part A. Manufacturing recommendations**

A.1 **Definitions**

A.1.1 **International name and proper name**

The international name should be “freeze-dried BCG 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**

Freeze-dried BCG vaccine is a freeze-dried preparation containing live bacteria derived from a culture of the bacillus of Calmette and Guérin, known as BCG, intended for intradermal injection. The name of the freeze-dried vaccine intended for percutaneous vaccination, should be “freeze-dried BCG vaccine, percutaneous”. The preparation should satisfy all the recommendations formulated below.
A.1.3 International Reference Preparations and Reference Reagents

The First WHO International Reference Preparation for BCG vaccine was established in 1965 and the First WHO International Standard for PPD of *M. tuberculosis* was established in 1951. Because of the age of these preparations, the need for replacements has been recognized, especially for the First WHO International Reference Preparation for BCG vaccine which is a live bacterial preparation. WHO has initiated the development of replacements. These were presented to the WHO Expert Committee on Biological Standardization in 2009 and 2010 as candidates for the First WHO International Reference Reagents for BCG vaccines of substrain Danish 1331, Tokyo 172-1 and Russian BCG-I (25, 26). These materials are available through the WHO web site (http://www.who.int/entity/bloodproducts/catalogue/BlooFeb2013.pdf). These International Reference Reagents cover the major proportion of BCG vaccine strains currently used in production. The establishment of substrain Moreau-RJ as the WHO International Reference Reagent for BCG vaccine is currently in progress and is scheduled for submission to the Committee in 2012 for adoption. These preparations are intended as International Reference Reagents, if required, for:

- periodical consistency monitoring of quantitative assays such as viability estimates (such as culturable particle count and modified ATP assays);
- residual virulence/local reactogenicity assays and protection assays in animal models for nonclinical evaluation.

They are also intended:

- as reference BCG substrains;
- for identity tests using multiplex polymerase chain reaction (PCR) as included in the collaborative study or in other molecular biology techniques.

The National Institute for Biological Standards and Control (NIBSC), England, distributes the WHO International Reference Reagents for BCG vaccines.

A.1.4 Terminology

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

**Final bulk**: the homogeneous finished liquid vaccine present in a single container from which the final containers are filled, either directly or through one or more intermediate containers derived from the initial single container.

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

**In-house reference:** a batch of vaccine prepared from the same BCG strain as the tested vaccine and used in parallel to the vaccine tested in:

- quantitative assays such as viability estimates (such as culturable particle count and modified ATP assays);
- residual virulence assays.

**Master seed lot:** a bacterial suspension of a single substrain originated from the bacillus of Calmette and Guérin that has been processed as a single lot and is of uniform composition. A seed lot should be maintained in the freeze-dried form stored at –20 °C or below (in the liquid form it is stored at –80 °C or below) in order to maintain viability. In each manufacturing establishment, a master seed lot is that from which material is drawn for inoculating media for the preparation of working seed lots or single harvests.

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

**Working seed lot:** a quantity of bacterial organisms of a single substrain derived from the master seed lot by growing the organisms and maintaining them in aliquots in the freeze-dried form stored at –20 °C or below (in the liquid form stored at –80 °C or below). The working seed lot should be prepared from the master seed lot by as few cultural passages as possible (e.g. 3–6 passages from the master seed lot), having the same characteristics as the master seed lot and intended for inoculating media for the preparation of single harvests.

### A.2 General manufacturing recommendations

The general manufacturing recommendations for manufacturing establishments contained in WHO’s Good manufacturing practices: main principles for pharmaceutical products (27) and Good manufacturing practices for biological products (28) should apply to establishments manufacturing BCG vaccine. In addition, the compliance with current good manufacturing practices should apply with the addition of the following.

Details of standard operating procedures for the preparation and testing of BCG vaccines adopted by the manufacturer, together with evidence of appropriate validation of each production step, should be submitted for the approval of the NRA. As required, proposals for the modification of manufacturing and control methods should also be submitted for approval to the NRA before they are implemented.

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

The degree of consistency in producing satisfactory final lots is an important factor in judging the efficacy and safety of a particular manufacturer’s product.

The data that should be considered in determining the consistency of production should include the results obtained with consecutive vaccine lots when tested as described in Part A, section 6 (e.g. the test for viability in Part A, section 6.7, and the thermal stability test in Part A, section 6.8).

More than two consecutive vaccine lots should have been satisfactorily prepared before any vaccine from a given manufacturer, or resulting from a new method of manufacture, is released. In subsequent routine production, if a specified proportion of vaccine lots or a specified number of consecutive vaccine lots fails to meet the requirements, the manufacture of BCG vaccine should be discontinued and should not be resumed until a thorough investigation has been made and the cause or causes of the failures determined to the satisfaction of the NRA.

Conventionally, production of BCG vaccine should take place in a dedicated area, completely separate from areas used for production of other medicines or vaccines, and using dedicated separate equipment. Such areas should be so situated and ventilated that the hazard of contamination is reduced to a minimum. No animals should be permitted in the vaccine production areas. Tests for the control of vaccine that require cultures to be made of contaminating microorganisms should be carried out in a completely separate area. Tests in which animals are used should also be carried out in a completely separate area.

For the purposes of these requirements, the processes of vaccine production that should take place in dedicated facilities are all operations up to and including the sealing of the vaccine in the final containers.

In some countries, the production of BCG vaccine – although isolated – is carried out in a building in which other work takes place. This should be done only after consultation with, and with the approval of, the NRA. If production takes place in part of a building, the work carried out in other parts of the building should be of such a nature that there is no possibility of cross-contamination with the BCG vaccine.

No cultures of microorganisms other than the BCG vaccine strain approved by the NRA for vaccine production should be introduced into the manufacturing areas. In particular, no strains of other mycobacterial species, whether pathogenic or not, should be permitted in the BCG vaccine production area.
BCG is susceptible to sunlight. Therefore, the procedures for the preparation of the vaccine should be so designed that all cultures and vaccines are protected from direct sunlight and ultraviolet light at all stages of manufacture, testing and storage, until the vaccine is issued.

BCG vaccine should be produced by a staff consisting of healthy persons who do not work with other infectious agents; in particular, they should not work with virulent strains of *M. tuberculosis*, nor should they be exposed to a known risk of tuberculosis infection. Precautions should also be taken to ensure that no worker should be employed in the preparation of BCG vaccine unless he or she has been shown by medical examination to be free from TB. The scope and nature of the medical examination should be at the discretion of the NRA. It may include a radiological examination and/or a validated immunological blood assay that should be repeated at intervals or when there is reason to suspect illness. The frequency of radiological examination should be at the discretion of the NRA, taking into consideration the incidence of TB in the country.

It is advisable to keep radiation exposure to a minimum, but the examination should be of sufficient frequency to detect the appearance of early active TB. It is estimated that, if workers in BCG vaccine laboratories were given one or two conventional X-ray examinations of the chest each year, not using fluoroscopic methods, and if the best available techniques were employed to minimize the radiation dose, the doses received would be considerably lower than the maximum permissible doses for workers occupationally exposed to radiation that have been set by the International Commission on Radiological Protection (29, 30).

Should an examination reveal signs of TB or suspected TB in a worker, he or she should no longer be allowed to work in the production areas and the rest of the staff should be examined for possible TB infection. In addition, all cultures should be discarded and the production areas decontaminated. If it is confirmed that the worker has TB, all vaccine made while he or she was in the production areas should be discarded, and all distributed batches should be recalled.

Persons not normally employed in the production areas should be excluded from them unless, after a medical examination, including radiological examination, they are shown to be free from TB. In particular, persons working with mycobacteria other than the BCG seed strain should be excluded at all times.

A.3  Control of source materials

A.3.1  Seed lot system

The production of vaccine should be based on the seed lot system. A seed lot prepared from a strain approved by the NRA (see Part D, section 1.1) should be prepared under conditions satisfying the requirements of Part A, sections 2, 3 and 4.
The BCG vaccine strain used should be identified by historical records that include information on its origin and subsequent manipulation. It would be preferable for the master seed lot to have protection proven clinically through clinical studies on a batch derived from it by a production process that is representative of the commercial process. It is also recommended to use a batch derived from such a clinically “validated” seed lot as an in-house reference in the laboratory to help ensure consistency in production.

If a working seed lot is being used, the total number of passages for a single production harvest should not exceed 12, including the passages necessary for preparing the working seed lot.

A.3.2 Tests on seed lot
A.3.2.1 Antimicrobial sensitivity test
An antimicrobial sensitivity test should be carried out as part of the ongoing characterization of BCG vaccine strains. It would be appropriate to test this property at the level of master or working seed lot.

A.3.2.2 Delayed hypersensitivity test
When a new working seed lot is established, a suitable test for delayed hypersensitivity in guinea-pigs is carried out; the vaccine is shown to be not significantly different in activity from the in-house reference.

A.3.2.3 Identity test
The bacteria in the master and working seed lots are identified as M. bovis BCG using microbiological techniques (e.g. morphological appearance of the bacilli in stained smears and the characteristic appearance of the colonies grown on solid media). Manufacturers are encouraged to carry out the test using molecular biology techniques (e.g. PCR test) to identify the specific substrain of BCG. The techniques will also provide relevant information to ensure genetic consistency in production, from master seed through working seed and to final product (4).

A.3.2.4 Test for bacterial and fungal contamination
Each master and working seed lot should be tested for bacterial and fungal contamination by appropriate tests, as specified in Part A, section 5.2 of General requirements for the sterility of biological substances (31), or by the validated methods approved by the NRA.

A.3.2.5 Test for absence of virulent mycobacteria
The test for absence of virulent mycobacteria, described in Part A, section 4.2.3, should be made in at least 10 healthy guinea-pigs injected with a quantity of
vaccine not less than 50 single human doses and should be observed for at least six weeks. If none of the animals shows signs of progressive TB and at least 90% survive the observation period (i.e. should one of the 10 animals die), the seed lot should be considered to be free from virulent mycobacteria.

If more than 10% of the guinea-pigs die during the observation period (i.e. should two out of 10 animals die) and freedom from progressive TB disease is verified, the test should be repeated on at least 10 more guinea-pigs. On the second occasion, the seed lot passes the test if not more than 10% of the animals die during the observation period (i.e. should one of the 10 animals die) and the autopsy does not reveal any sign of TB.

A.3.2.6 Test for excessive dermal reactivity
The test for excessive dermal reactivity, described in Part A, section 6.4.2, should be made in six healthy guinea-pigs, each weighing not less than 250 g and having received no treatment likely to interfere with the test. Each guinea-pig should be injected intradermally, according to a randomized plan, with 0.1 ml of the reconstituted vaccine and of vaccine dilutions 1:10 and 1:100. The same dilutions of the appropriate international Reference Reagent or in-house reference should be injected into the same guinea-pigs at randomly selected sites. The guinea-pigs should be observed for at least four weeks. The vaccine complies with the test if the reactions it produces at the sites of injection are not markedly different from those produced by the appropriate international Reference Reagent or in-house reference.

A.3.3 Production culture medium
The production culture medium should contain no substances known to cause toxic or allergic reactions in humans. The use of material originating from animals should be discouraged. However, if constituents derived from animals are necessary, approval of the NRA should be sought and the materials should comply with current policy on TSEs (32–37). A risk assessment for TSE would need to be included for the materials of the culture medium. WHO's revised Guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products (32) provide guidance on risk assessments for master and working seeds and should be consulted. Substances used in that medium should meet such specifications as the NRA may prescribe.

A.4 Control of vaccine production
A.4.1 Control of single harvests
All cultures should be examined visually, and any that have grown in an uncharacteristic manner should not be used for vaccine production.
A.4.2  Control of final bulk

A.4.2.1 Final bulk

The final bulk should be prepared from a single harvest or by pooling a number of single harvests.

A.4.2.2 Test for bacterial and fungal contamination

The final bulk should be tested for bacterial and fungal contamination by appropriate tests as specified in Part A, section 5.2 of the General requirements for the sterility of biological substances (31), or by the validated methods approved by the NRA. No vaccine lot should be passed for use unless the final bulk has been shown to be free from such contamination.

A.4.2.3 Test for absence of virulent mycobacteria

The test for absence of virulent mycobacteria should be carried out on each final bulk or final lot.

At least six healthy guinea-pigs, all of the same sex, each weighing 250–400 g should be used. They should not have received any treatment or diet, such as antibiotics, that is likely to interfere with the test. A sample of the final bulk intended for this test should be stored at 4 °C for not more than 72 hours after harvest.

A dose of BCG organisms corresponding to at least 50 single human doses of vaccine intended for intradermal injection should be injected into each guinea-pig by the subcutaneous or intramuscular route. The guinea-pigs should be observed for at least six weeks. If, during that time, they remain healthy, gain weight, show no signs of progressive TB and not more than one dies, the final bulk should be considered to be free from virulent mycobacteria.

At the end of the observation period, the animals should be killed and examined postmortem for macroscopic evidence of progressive TB disease. Similarly, any animals that die before the end of the observation period should be subjected to a postmortem examination.

Should one third of the guinea-pigs die (i.e. should two out of six animals die) during the observation period (and freedom from progressive TB disease is verified), the test should be repeated on at least six more guinea-pigs. On the second occasion, the vaccine lot passes the test if not more than one

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1 When a more concentrated vaccine, intended for administration by the percutaneous route, is tested, a dilution factor approved by the NRA should be applied so that the mass of BCG injected corresponds to at least 50 human doses of intradermal vaccine.
animal dies during the observation period and the autopsy does not reveal any sign of TB.

Should a vaccine lot fail to satisfy the requirements of this test because animals die from causes other than TB, the procedure to be followed by the manufacturer should be determined with the approval of the NRA.

If signs of TB disease are seen, the vaccine lot should be rejected, all subsequent vaccine lots should be withheld, and all current vaccine stocks should be held pending further investigation. The manufacture of BCG vaccine should be discontinued and it should not be resumed until a thorough investigation has been made and the cause or causes of the failure determined and appropriate actions have been taken. Production should be allowed to resume only upon the approval of the NRA.

A.4.2.4 Test for bacterial concentration

The bacterial concentration of the final bulk should be estimated by a validated method approved by the NRA and should have a value within a range approved by the NRA (see Part D, section 1.2).

Based on manufacturers’ experience, the opacity method is the method of choice. The International Reference Preparation of Opacity,\(^2\) or an equivalent Reference Preparation approved by the NRA, may be employed in comparative tests.

Clumping issues should be considered during validation of the assay.

A.4.2.5 Test for number of culturable particles

The number of culturable particles on a solid medium of each final bulk should be determined by an appropriate method approved by the NRA. Alternatively, a bioluminescence or other biochemical method can be used (38, 39), provided that the method is properly validated against the culturable particle test for the production step in question. If properly validated, such tests can be used as equivalent methods. Regular calibration with the reference method as agreed with the NRA would be relevant.

The medium used in this test should be such that the number of culturable particles may be determined at an optimal time point (usually 3–5 weeks) after the medium has been inoculated with dilutions of the vaccine.

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\(^2\) The International Reference Preparation of Opacity is in the custody of the NIBSC, Potters Bar, England, which supplies samples on request.
There are various methods of determining the number of culturable particles in BCG vaccine, and it is essential that only one culture method be used for all the vaccine lots produced by a manufacturer (5). It is also desirable for assay validation that the clumping issue should be considered and that tests should be carried out in parallel with the appropriate international Reference Reagent or in-house reference, e.g. the same vaccine production that has been used in clinical trials and has assured safety (including immunogenicity) and efficacy.

A.4.2.6 Substances added to the final bulk

Substances used in preparing the final bulk should meet such specifications as the NRA may prescribe. In particular, the NRA should approve the source(s) of any animal-derived raw materials which should comply with the WHO Guidelines on tissue infectivity distribution in transmissible spongiform encephalopathies (34).

Substances added to improve the efficiency of the freeze-drying process or to aid the stability of the freeze-dried product should be sterile and of high and consistent quality, and should be used at suitable concentrations in the vaccine.

A.5 Filling and containers

The general requirements concerning filling and containers given in Good manufacturing practices for biological products (28) should apply to vaccine filled in the final form.

The containers should be in a form that renders the process of reconstitution as simple as possible. Their packaging should be such that the reconstituted vaccine is protected from direct sunlight.

A.6 Control tests on final lot

Tests on the final lot should be performed after reconstitution, except for appearance and residual moisture tests. The diluent supplied or recommended for reconstitution should be used, unless such diluent would interfere with any of the tests, in which case some other suitable fluid should be used. The vaccine should be reconstituted to the concentration at which it is to be used for injection into humans; however, an exception may be made in the case of the test for absence of virulent mycobacteria (Part A, section 6.4.1), when a higher concentration of reconstituted vaccine may be necessary. It would be appropriate to monitor periodically the antimicrobial sensitivity in final lots.

A.6.1 Inspection of final containers

Every container in each final lot should be inspected visually, and those showing abnormalities should be discarded.
The appearance of the freeze-dried vaccine and the reconstituted vaccine should be described with respect to form and colour. If reconstitution with the product diluent does not allow for the detection of particulates, an alternative diluent may be used.

A.6.2 **Identity test**

An identity test should be performed on samples of the vaccine from each final lot. The identity test for final lots should be used to identify the product as BCG as approved by the NRA. The identity of each final lot of vaccine should be verified by the morphological appearance of the bacilli in stained smears and by the characteristic appearance of the colonies grown on solid media. A validated nucleic acid amplification technique (such as PCR) should preferably be used.

A.6.3 **Test for bacterial and fungal contamination**

Samples from each final lot should be tested for bacterial and fungal contamination by appropriate tests as specified in Part A, section 5.2 of the General requirements for the sterility of biological substances (31), or by the validated methods approved by the NRA.

A.6.4 **Safety tests**

A.6.4.1 **Test for absence of virulent mycobacteria**

Provided the test for virulent mycobacteria has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

If the test for the absence of virulent mycobacteria, applied to the final bulk, is unsatisfactory (and freedom from progressive TB disease is verified), it should be repeated with a sample of a final lot (see Part A, section 4.2.3).

A.6.4.2 **Test for excessive dermal reactivity**

Provided the test has been carried out with satisfactory results on the working seed lot and on at least three consecutive final lots produced from it, the test may be omitted on the final lot.

Historically the omission of the test with satisfactory results on five consecutive final lots has been accepted by the authorities (40).

A.6.5 **Test for bacterial concentration**

The total bacterial content of the reconstituted vaccine should be estimated for each vaccine lot by a validated method approved by the NRA, and should have a value within a range approved by the NRA (see Part D, section 1.2).
The estimation of total bacterial content may be made either directly, by determining the dry weight of organisms, or indirectly by an opacity method that has been calibrated in relation to the dry weight of the organisms.

The clumping issue should be considered during validation of the assay.

A.6.6 Test for residual moisture

The average moisture content of a freeze-dried vaccine should be determined by a validated method accepted by the NRA. Values should be within limits of the preparations shown to be adequately stable in the stability studies of the vaccine.

A.6.7 Tests for viability

A.6.7.1 Test for number of culturable particles

The number of culturable particles of each final lot should be determined by an appropriate method approved by the NRA (see Part A, section 4.2.5). The viable count should have a value within a range approved by the NRA that should not be wider than a fourfold difference between the lower and upper levels of the specification for numbers of culturable particles (see Part D, section 1.2). By comparison with the results of the test for number of culturable particles carried out on final bulk, as described in Part A, section 4.2.5, the percentage survival on freeze-drying may be calculated and this value should be not less than one approved by the NRA. The appropriate international Reference Reagent or in-house reference should be used for every test in order to validate the assay.

The purpose of including the appropriate international Reference Reagent or in-house reference is to have a check on the quality and consistency of the culture medium and the accuracy of the technique used for the determination of the number of culturable particles. It is not intended to adjust the count of the vaccine by comparison with the Reference Preparation.

The clumping issue should be considered during validation of the assay.

The survival rate after freeze-drying is usually not less than 20%.

A.6.7.2 Rapid test for viability

As an alternative to the colony counting method, a bioluminescence or other biochemical method can be used provided that the method is properly validated against the culturable particle test for the production step in question. If properly validated, such tests may be considered by the NRA to replace the culturable particle test.
The bioluminescence reaction occurring in fireflies depends on the presence of adenosine triphosphate (ATP), luciferin luciferase, oxygen and magnesium ions. This reaction can be reproduced in vitro by mixing these components. If all components except ATP are present in excess, the amount of light emitted is proportional to the amount of ATP coming from the vaccine.

Since ATP is present in all living cells and is immediately destroyed when the cell dies, ATP is a reliable marker for living cells.

Studies have shown that, if properly validated, measurement of ATP using the bioluminescence reaction can be used to estimate the viable count of freeze-dried BCG vaccine within 1–2 days as accurately as other, more time-consuming methods, once the mean content of ATP per culturable particle has been estimated for a given vaccine production.

### A.6.8 Thermal stability test

The thermal stability test is part of the characterization and consistency demonstration of vaccine production. The requirement for this test should be at the discretion of the NRA and, if required, each final lot should be tested for thermal stability by a validated method approved by the NRA. If the production consistency is demonstrated, this test may be omitted on the final lot subject to NRA approval (6).

If performed, the test should involve the determination of the number of culturable particles before and after the samples have been held at appropriate temperatures and for appropriate periods.

For example, the thermal stability test may be carried out by taking samples of the vaccine and incubating them at 37 °C for 28 days.

The percentage decrease in the number of culturable particles is then compared with that of samples of the same vaccine lot stored at 2–8 °C. The number of culturable particles in the vaccine after heating should be not less than 20% of that stored at 2–8 °C (41). The absolute value should be approved by the NRA. The viability test should also be performed with the appropriate international Reference Reagent or in-house reference for checking validity of the assay. One method of determining the number of culturable particles should be adhered to, as suggested in Part A, section 4.2.5.

The purpose of including the appropriate international Reference Reagent or in-house reference is to check the quality and consistency of the medium used for determination of the number of culturable particles. It is not intended to adjust the count of the vaccine by comparison with the Reference Preparation.
All manufacturers should keep their product for the approved storage period and should determine the number of culturable particles from time to time to demonstrate that the number is being maintained at an adequate level.

In some countries, the thermal stability test is carried out only after the vaccine has been stored for 3–4 weeks after freeze-drying, since it is considered that the degree of stability during the first three weeks may not be related to the long-term stability of the product.

As a guide to stability, some manufacturers of freeze-dried BCG vaccine determine the residual moisture content of the final vaccine, since failure to achieve a certain degree of desiccation results in an unstable product. However, such a test cannot be regarded as an alternative to tests involving the determination of the number of culturable particles.

A.7 **Records**

The recommendations in section 8 of Good manufacturing practices for biological products (28) should apply.

Written records should be kept of all seed lots, all cultures intended for vaccine production, all single harvests, all final bulk vaccines, and all vaccine in the final containers produced by the manufacturing establishments, including all tests irrespective of their results.

The records should be of a type approved by the NRA. An example of a suitable protocol is given in Appendix 2.

A.8 **Retained samples**

The recommendations in section 9.5 of Good manufacturing practices for biological products (28) should apply.

It is desirable that samples should be retained for at least one year after the expiry date of the final lot.

A.9 **Labelling**

The recommendations in section 7 of Good manufacturing practices for biological products (28) should apply, including the following guidance.

The label, and/or the packaging insert in some countries, printed on or affixed to each container should show the volume and nature of the diluent. Also, this label, or the label on the carton holding several final containers, or the leaflet accompanying the containers, should carry the following additional information:

- the fact that the vaccine fulfils the requirements of this document;
- instructions for use of the vaccine and information concerning contraindications and the reactions that may follow vaccination;
- the volume and nature of the diluent to be added to reconstitute
  the vaccine, specifying that only the diluent supplied by the
  manufacturer should be used;
- the conditions recommended during storage and transport, with
  information on the reduced stability of the vaccine if exposed to
  temperatures higher than that stated on the label;
- warnings that the vaccine should be protected from direct sunlight;
- a statement that the reconstituted vaccine should be used as soon as
  possible, or should be stored at 2–8 °C, protected from direct sunlight
  and used within six hours (42);
- information on antimicrobial sensitivity.

The label for the diluent should state “Reconstituting fluid for BCG
vaccine [proprietary name]”.

A.10 Distribution and transport

The recommendations given in section 8 of Good manufacturing practices for
biological products (28) should be followed, along with the guidance provided
in Safe vaccine handling, cold chain and immunizations (43). Further guidance
is provided in Model guidance for the storage and transport of time- and
temperature-sensitive pharmaceutical products (44).

It is desirable that samples should be retained for at least one year after
the expiry date of the final lot.

A.11 Stability, storage and expiry date

A.11.1 Stability testing

Adequate stability studies form an essential part of vaccine development. The
recommendations provided in WHO’s Guidelines for stability evaluation of
vaccines should be followed (46). Stability testing should be performed at different
stages of production if stored for a given time period, namely as appropriate for
single harvests or pool of single harvests, final bulk or final lot. Stability-indicating
parameters should be defined or selected appropriately according to the stage of
production. It is advisable to assign a storage period to all in-process materials
during vaccine production, particularly intermediates such as single harvests and
final bulk, and a shelf-life period to the final lots.

BCG vaccines require special precautions to ensure sufficient stability.
In this connection the most important measures are lyophilization, the use of an
effective stabilizer, and proper sealing of vaccine containers.
Historically the use of ampoules sealed under vacuum was the most common practice for increasing stability. However, vacuum-sealing is difficult compared to sealing in the presence of inert gas. There were no significant differences between BCG vaccines sealed under vacuum and under nitrogen or carbon dioxide at either 4°C or 37°C (41). Manufacturers now prepare BCG vaccines in vials/ampoules and, under well-validated conditions, the product is adequately stable.

A.11.2 Storage conditions

The Guideline for establishing or improving primary and intermediate vaccine stores (47) should apply.

Storage conditions should be based on stability studies and approved by the NRA. Before being distributed by the manufacturing establishment, or before being issued from a depot for the storage of vaccine, all vaccines in their final containers should be stored constantly at 2–8°C (41, 48) and vaccine diluents should be stored as recommended by the manufacturer. Freeze-dried BCG vaccines, regardless of their substrain, are sensitive to ultraviolet and fluorescent light. They should be protected from direct sunlight (41).

BCG vaccines are sensitive to light as well as to heat. Normally, these vaccines are supplied in vials/ampoules made from dark brown glass, which gives them some protection against light damage, but care should still be taken to keep them covered and protected from strong light at all times (48).

Freeze-dried BCG vaccines may be kept frozen at −15°C to −25°C if cold chain space permits, but this is neither essential nor recommended (41).

Precautions should also be taken to maintain the vaccine during transport and up to the time of use at the temperature and under the storage conditions recommended by the manufacturer.

A.11.3 Expiry date

The expiry date should be approved by the NRA and should be based on the stability of the final product, as well as on the results of the stability tests referred to in section A.11.1 above. It is established for each batch by adding the shelf-life period to the date of manufacture. Most freeze-dried BCG vaccines are stable at temperatures of 2–8°C for at least two years (41) from the date of manufacture. The storage of final product at −20°C to extend the shelf-life should be validated.

A.11.4 Expiry of reconstituted vaccine

Stability studies should be undertaken on reconstituted vaccine. Freeze-dried BCG vaccines become much more heat-sensitive after they have been
reconstituted with diluent (41). After multidose containers of freeze-dried BCG have been reconstituted, the vaccine should be used as soon as possible. Any reconstituted vaccine remaining should be stored at 2–8 °C until used, and the expiry time should be defined by stability studies (4, 42, 45).

Part B. Nonclinical evaluation of BCG vaccines

Details on the design, conduct, analysis and evaluation of nonclinical studies are available in the WHO Guidelines on nonclinical evaluation of vaccines (49).

Nonclinical testing of a new strain (i.e. a strain derived by selection from existing BCG strains in Appendix 1) or of a strain from a new manufacturer of a BCG vaccine is a prerequisite for initiation of clinical studies in humans. Nonclinical testing includes immunogenicity, protection studies (proof of concept) and safety testing in animals. The vaccine lots used in nonclinical studies should be adequately representative of the formulation intended for clinical investigation and, ideally, should be the same lots, manufactured according to current good manufacturing practice (cGMP), as those used in clinical studies. If this is not feasible, the lots used clinically should be comparable to those used in the nonclinical studies with respect to potency, stability and other characteristics of quality. The technical manufacturing consistency lots may often be used for these purposes.

New manufacturers of BCG vaccine for human use will need to refer to the range of nonclinical safety and characterization tests that are recommended for existing licensed BCG vaccines. Although there is currently no requirement for additional nonclinical testing beyond that already described for licensed BCG vaccines, the development of new variants of BCG, the potential for new fermentation technologies and the possibility of novel live vaccines against TB have shown that additional nonclinical studies beyond that required for licensed BCG vaccine can be helpful in demonstrating that a new BCG product has satisfactory nonclinical efficacy, safety and stability.

Guideline example on protective potency testing: Hartley guinea-pigs are used for potency testing. The guinea-pigs are vaccinated with a small amount of BCG (~10³ colony-forming units (CFU)). Eight weeks after the vaccination, the guinea-pigs are challenged with virulent *M. tuberculosis* H37Rv (ATCC 27294) by the pulmonary route with a low dose (10–15 CFU) per animal. Five weeks after the infection, the guinea-pigs are killed and the spleen and the lung lobes are removed. These organs are homogenized separately. Appropriate dilutions are inoculated on to duplicate solid medium and incubated at 37 °C for three weeks. The number of *M. tuberculosis* H37Rv colonies is counted, and is expressed as mean log₁₀ CFU per tissue. The CFU results are compared between the vaccinated and non-vaccinated groups (50).
If there are two pharmacologically relevant species for the clinical candidate (one rodent and one non-rodent), both species should be used for short-term (up to one month duration) toxicology studies. If the toxicological findings from these studies are similar in both species, longer-term studies in one species are usually considered sufficient; the rodent species should be considered unless there is a rationale for using non-rodents. Studies in two non-rodent species are not appropriate. Other in vivo studies should address both potency (such as tuberculin sensitivity and immunological tests) and safety issues (such as tests for excessive dermal reactivity and absence of virulent mycobacteria) of the classical BCG vaccines.

It may be of benefit for new BCG vaccine developers to consider the points raised in recent meetings establishing recommendations for new live vaccines against TB (51, 52).

**Part C. Clinical evaluation of BCG vaccines**

Clinical trials should adhere to the principles described in the Guidelines for good clinical practice (GCP) for trials on pharmaceutical products (53) and the general principles described in WHO's Guidelines on clinical evaluation of vaccines: regulatory expectations (54). All clinical trials should be approved by the relevant NRAs and local ethics committees. Continued licence of BCG vaccines should be viewed in the light of ongoing post-marketing data on the safety, immunogenicity and effectiveness of BCG vaccines in the target population.

Part C considers the provision of clinical data required (a) when a new candidate “classical” BCG vaccine derived from (the same master seed of) one of the recognized strains (see Appendix 1) is developed; (b) when there have been major changes to the manufacturing process of an established vaccine, including preparation of a new master seed lot of an established strain; (c) when technology transfer of existing vaccine is planned to a new manufacturer; and (d) when revalidation of existing vaccines used in national immunization programmes is considered.

Vaccines manufactured using a “new strain” (i.e. a strain derived by selection from existing BCG strains in Appendix 1) should require a full clinical development programme that provides evidence of safety, efficacy and the reactogenicity profile in all target age-groups.

Other vaccines against *M. tuberculosis* derived from *M. bovis* or other mycobacterial strains cannot be considered as BCG. They would require a full clinical development programme and are not included here.

**C.1 General considerations**

**C.1.1 Comparative or placebo-controlled clinical trials**

It would not be considered ethical to conduct a placebo-controlled trial of protective efficacy of a BCG vaccine in a TB-endemic area, particularly in
Infants. A comparative trial with a licensed, or internationally accepted (WHO prequalified), BCG vaccine could be accepted.

C.1.2 Value of PPD response

It is recognized that the response to tuberculin PPD is not an indicator of a protective immune response. Nonetheless this has been used for more than 50 years to indicate a cellular immune response to an infection with *M. tuberculosis* or as evidence of “successful” BCG vaccination. At best, a PPD reaction is an indicator of exposure to antigens of TB, and the generation of a cellular immune response. Thus, it can be used in a PPD-naive population as an indicator of an immune response to the BCG vaccine (55). Other immunological measures may be more closely related to *M. tuberculosis* infection or vaccination, but currently none has been agreed as a correlate of protection from infection or disease.

C.1.3 BCG in HIV-infected infants

A very important safety consideration with regard to vaccination policy is to establish, during clinical trials, the potential for disseminated BCG disease in immunocompromised children. In this regard, the use of BCG vaccines at birth should follow the recommendations from the GACVS (13, 14). The GACVS recommendations consider the policies for immunization exclusion of infants known to be infected with HIV, infants symptomatic for HIV infection, and those infants born to mothers known to be HIV-infected and who therefore may be infected.

C.1.4 Post-vaccination reactions and complications

Vaccines intended for intradermal or percutaneous injection should be given strictly intradermally or percutaneously, and vaccinators should be trained accordingly. Incorrect vaccination technique can result in adverse reactions, including discharging ulcers, abscesses and keloid scars.

Current BCG vaccines have a known reactogenicity profile after intradermal inoculation (56). Local reaction at the vaccination site is normal after a BCG vaccination. It may take the form of a nodule that, in many cases, will break down and suppurate. The reaction developing at the vaccination site usually subsides within 2–5 months and in practically all children leaves a superficial scar of 2–10 mm in diameter. The nodule may persist and ulcerate. Swelling of regional lymph nodes may also be seen, and this may be regarded as a normal reaction, but the size should be limited.

Keloid and lupoid reactions may occur at the site of the vaccination. Children with such reactions should not be revaccinated. Inadvertent subcutaneous injections produce abscess formations and may lead to ugly retracted scars. Among the major complications, suppurative lymphadenitis has been observed.
In the case of certain vaccines, it has been revealed that there is a strong correlation between the incidence of these complications in newborns and the number of culturable particles in the vaccine.

The concentration of the vaccine should be shown to be effective and tolerated in the age groups for which the vaccine is intended.

A reduction of the dose for newborns may be based on the evidence and approved by the NRA (57).

The NRA should issue guidelines for the treatment of complications.

C.2 Special considerations

This section is limited to the clinical development of new “classical” BCG vaccines manufactured following these Recommendations and using strains of BCG that are derived from (the same master seed of) one of the strains recognized in Appendix 1.

The use of comparative studies with a licensed BCG vaccine can provide evidence of the similarity of safety and immune responses to a new classical BCG vaccine product.

The target population for the vaccine would be newborns or infants according to current recommendations on the use of BCG vaccines.

The nonclinical expectations for a new classical BCG vaccine are outlined in Part B.

For such a new classical BCG vaccine, these nonclinical studies should be conducted in comparison to an existing licensed BCG vaccine, preferably derived from the same BCG substrain. It would be expected that the results of preclinical studies would be similar for the new vaccine product and for the comparator.

The clinical development programme should ideally be designed to show the safety and protective efficacy of the vaccine. However, for such a new classical BCG vaccine product, comparative studies with an existing licensed BCG vaccine, using immunological responses as a marker for efficacy, may be acceptable to the responsible NRA.

Comparable PPD response (proportion of PPD converters, intensity of response) may be acceptable.

Clinical studies should provide evidence of safety in all the potential target populations, including those with a high incidence of diseases that may affect the safety or efficacy of the new vaccine product. In phase I and phase II studies this should include evaluation of:

- safety and reactogenicity in healthy adults (comparative);
- end-points;
- safety and reactogenicity – can include healthy HIV-infected adults;
- immune responses – non-inferior PPD response, and may include other immunological markers.

These studies are difficult to interpret as adults will most likely have received BCG vaccination at birth. Dose-finding studies may be considered unnecessary for these vaccines. The safety in HIV-infected individuals and in infants needs to be considered.

Dose-finding and age de-escalation can be included in these studies, but review at each step by a suitable independent safety committee should be considered. In phase III studies evaluation should be made of:

- safety and reactogenicity in infants (comparative)
- end-points
- safety and reactogenicity
- non-inferior PPD immune response.

**Post-marketing risk management**

As it may not be practically possible to evaluate protective efficacy for a new classical BCG vaccine, the responsible NRA in the country of manufacture should require post-marketing surveillance activities for safety and effectiveness in a suitable environment. Sentinel surveillance sites in an endemic country may be considered.

In the past, the responsible NRA of a country of manufacture required a demonstration that adequate control of BCG vaccine had been achieved, by arranging for studies of some of the final lots to be performed at regular intervals in children.

Studies of immunological responses to *M. tuberculosis* antigens should be made, such as sensitivity to tuberculin. In at least 100 tuberculin-negative persons per year, records of tuberculin-induced reaction (distribution of tuberculin reactions by size) by a defined dose of tuberculin\(^3\) in vaccines, local skin lesions (nature and size of reaction at injection site), and the occurrence of untoward vaccination reactions should be obtained. Such tests should be performed in parallel on two or more vaccine lots in the same population group, one of the vaccine lots being preferably a reference vaccine.

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\(^3\) An intradermal test with a dose of tuberculin equivalent to 5 IU of tuberculin PPD is suitable. A description of an appropriate method and a design for a study to assess BCG vaccines in humans are available on application to the World Health Organization, 1211 Geneva 27, Switzerland.
C.3 Post-marketing surveillance

The NRA in the country of manufacture may require periodic updates of reports on BCG safety and immunogenicity.

C.3.1 BCG vaccine used in a national immunization programme

As in all immunization programmes, the adverse events following immunization with BCG vaccines should be monitored and recorded.

The following events are important after BCG vaccination (58):

- injection site abscesses
- BCG lymphadenitis
- disseminated BCG diseases
- osteitis/osteomyelitis.

Appropriate training of health-care workers is important as some medical incidents can be related to immunization even if they have a delayed onset.

C.3.2 WHO prequalified BCG vaccines

Prequalified vaccines may be used in a wide range of countries worldwide. Periodic safety update reports supplied to WHO should include specific analysis of countries where the vaccine has been used.

Part D. Guidelines for NRAs

D.1 General

The general recommendations for NRAs provided in the Guidelines for national authorities on quality assurance for biological products (59) should apply. These specify that no new biological substance should be released until consistency of manufacturing and quality as demonstrated by a consistent release of batches has been established. The detailed production and control procedures, as well as any significant change in them that may affect the quality, safety or efficacy of BCG vaccine, should be discussed with and approved by the NRA. For control purposes, the NRA should obtain the WHO International Reference Reagents as comparators for potency-related testing and, where necessary, should establish national working Reference Preparation(s) calibrated against the international reference.

In addition, the NRA should provide a reference vaccine or approve one used by a manufacturer, and should give directions concerning the use of the reference vaccine in specified tests. The NRA should also give directions to
manufacturers concerning the BCG substrain to be used in vaccine production, the total content of bacteria, the number of culturable particles, and the stability required of the vaccine, and should specify the requirements to be fulfilled by the manufacturer in accordance with the provisions of Part A of this document, including those for consistency of quality in respect of the points referred to in Part A, section 2.

D.1.1 **BCG vaccine strain**

The substrain of BCG (maintained in the form of a seed lot) used in the production of vaccine should be derived from the original strain maintained by Calmette and Guérin and should be identified by historical records that include information on its origin and subsequent manipulation. On the basis of cultures and biochemical and animal tests, the BCG seed lot should show characteristics that conform to those of BCG and generally differ from those of other mycobacteria. The identity test should be supplemented by molecular biology techniques to identify the specific BCG substrain used. The seed lot should show consistency in the morphological appearance of colonies and genetic stability on serial subculture. It should also have been shown to yield vaccines that, upon administration by intradermal injection to children and adults, induce relevant immunological responses to *M. tuberculosis* antigens, including sensitivity to tuberculin, and with a low frequency of untoward effects. In addition, the seed lot should have been shown to give adequate protection against TB in experimental animals in tests for protective potency.

D.1.2 **Concentration of BCG vaccine**

The concentration of BCG vaccine varies with different vaccine products and is dependent on a number of factors, such as the substrain of BCG used and the method of manufacture. It is therefore essential for each manufacturer, as well as for each method of manufacture, for the optimum potency of vaccine to be ascertained by trials in tuberculin-negative subjects (newborns, older children, and adults) to determine the response to vaccination in respect of the induction of relevant immunological responses to *M. tuberculosis* antigens – including sensitivity to tuberculin, the production of acceptable local skin lesions, and the occurrence of a low frequency of untoward reactions. As a result of such trials, the NRA should give directions to the manufacturer concerning the total bacterial content and the number of culturable particles required for the vaccine.

If a manufacturer changes its procedure for preparing BCG vaccine, and if the NRA considers that the change might affect the final product, it may be necessary to conduct further clinical trials in order to determine the optimum content of BCG organisms in the new product.
D.2 Release and certification

A vaccine lot should be released only if it fulfils the national requirements and/or Part A of these Recommendations. Before any vaccine lot is released from a manufacturing establishment, the recommendations for consistency of production provided in the Guidelines for national authorities on quality assurance for biological products (59) should be met. In addition, the general recommendations for NRAs provided in the Guidelines for independent lot release of vaccines by regulatory authorities (60) should be followed. A protocol based on the model given in Appendix 2, signed by the responsible official of the manufacturing establishment, should be prepared and submitted to the NRA in support of a request for release of a vaccine for use.

A statement signed by the appropriate official of the NRA (or authority as appropriate) 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 date of manufacture, 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 certificate should be based on the model given in Appendix 3. The purpose of the certificate is to facilitate the exchange of vaccines between countries.

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Several draft Recommendations were subsequently prepared by Dr M.M. Ho, National Institute for Biological Standards and Control, Potters Bar, England, with support from the drafting group of Dr M. Corbel, Milton Keynes, England; Dr R. Dobbelzaer, Lokeren, Belgium; Dr H.-N. Kang, World Health
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Following the meeting of the drafting group in March 2011, held in Potters Bar, England, draft Recommendations were updated taking into account comments received from:

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The draft Recommendations were posted on the WHO Biologicals web site for public consultation from 24 May to 23 June 2011. The WHO/BS/2011.2157 document was then prepared by Dr M.M. Ho, National Institute for Biological Standards and Control, Potters Bar, England; Dr H.-N. Kang, World Health Organization, Geneva, Switzerland; Dr J. Southern, Cape Town, South Africa; Dr K.B. Walker, National Institute for Biological Standards and Control, Potters Bar, England, taking into account comments received from the following reviewers:

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Further changes were made to WHO/BS/2011.2157 by the Expert Committee on Biological Standardization, resulting in the current document.

References


Appendix 1

History and genealogy of BCG substrains

Note: This diagram provides only a historical overview of the use of different substrains derived from BCG vaccine strain. It does not indicate any WHO "qualification" or "approval" of the strains or vaccines in the context of this document.

Appendix 2

Summary protocol for manufacturing and control of BCG vaccine

The following protocol is intended for guidance, and indicates the information that should be provided as a minimum by the manufacturer to the NRA. Information and tests may be added or omitted as required by the NRA, 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 recommendations of 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 NRA or NCL of the country in which the vaccine was produced stating that the product meets national requirements as well as Part A of the recommendations of this document published by WHO.

Summary information on the finished product (final lot)

International name: ________________________________
Trade name: ________________________________
Product licence (marketing authorization) number: ________________________________
Country: ________________________________
Name and address of manufacturer: ________________________________
Site of manufacture of final lot: ________________________________
Name and address of licence-holder
  (if different): ________________________________
BCG substrain: ________________________________
Authority that approved the BCG substrain: ________________________________
Date approved: ________________________________
Final bulk number: ________________________________
Volume of final bulk: ________________________________
Final product: ________________________________
Type of vaccine: Intradermal/Percutaneous/Other
Final lot number: ________________________________
Type of container: ________________________________
Number of doses per container: ____________________________
Number of filled containers in this final lot: ____________________________
Date of manufacture of final lot: ____________________________
Date on which last determination of the bacterial count was started, or date of start of period of validity: ____________________________
Shelf-life approved (months): ____________________________
Expiry date: ____________________________
Diluent: ____________________________
Storage conditions: ____________________________
Volume of single human dose: ____________________________
Volume of vaccine per container: ____________________________
Number of doses per container: ____________________________
Summary of the composition (include a summary of the qualitative and quantitative composition of the vaccine per human dose): ____________________________
Release date: ____________________________

Production information

A genealogy of the lot numbers of all vaccine components used in the formulation of the final product will be informative.

The following sections are intended for 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.

Control of source materials (section A.3)

The information requested below is to be presented on each submission. Full details on master and working seed lots are required on first submission only and whenever a change has been introduced.

Master seed lot

Origin of seed lot: ____________________________
Master seed lot number: ____________________________
Name and address of manufacturer: ____________________________
Passage level: ____________________________
Date of preparation of seed lot: ____________________________
Date of receipt of seed lot (if applicable): ____________________________
Date of reconstitution of seed lot ampoule: ____________________________
Date approved by the NRA: ____________________________
Working seed lot

Working seed lot number: ________________________________
Name and address of manufacturer: __________________________
Passage level: ________________________________
Date of reconstitution of seed lot ampoule: _______________________
Date approved by the NRA: ________________________________

Tests on working seed lot production (section A.3.2)

Antimicrobial sensitivity test (section A.3.2.1)

Method used: ________________________________
Date test started: ________________________________
Date test completed: ________________________________
Results: ________________________________

Delayed hypersensitivity test (section A.3.2.2)

Method used:

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Reference vaccine</th>
</tr>
</thead>
<tbody>
<tr>
<td>_______</td>
<td>_______</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dilutions injected:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>_______</td>
<td>_______</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Inoculation route:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>_______</td>
<td>_______</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Number of guinea-pigs given injection:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>_______</td>
<td>_______</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Observation period (specification):</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>_______</td>
<td>_______</td>
</tr>
</tbody>
</table>

Date test started: ________________________________
Date test completed: ________________________________
Result: ________________________________

Identity test (section A.3.2.3)

Method used: ________________________________
Date test started: ________________________________
Date test completed: ________________________________
Results: ________________________________

Test for bacterial and fungal contamination (section A.3.2.4)

Method used: ________________________________
Number of containers tested: ________________________________
Volume of inoculum per container: ________________________________
Volume of medium per container: ________________________________
Observation period (specification): ________________________________
Test for absence of virulent mycobacteria (section A.3.2.5)

Method used: ____________________________________________________________
No. of human doses injected per guinea-pig: _________________________________
Inoculation route: _______________________________________________________  
No. of guinea-pigs given injection: _________________________________________  
Weight range of guinea-pigs: _____________________________________________  
Observation period (specification): ________________________________________  
Date test started: _______________________________________________________  
Date test completed: ___________________________________________________  
Health of animals during test: ____________________________________________  
Weight gains (losses): ____________________________________________________  
Result: _________________________________________________________________

Test for excessive dermal reactivity (section A.3.2.6)

Method used: 

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Reference vaccine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilutions injected:</td>
<td></td>
</tr>
<tr>
<td>Inoculation route:</td>
<td></td>
</tr>
<tr>
<td>No. of guinea-pigs given injection:</td>
<td></td>
</tr>
<tr>
<td>Observation period (specification):</td>
<td></td>
</tr>
<tr>
<td>Date test started:</td>
<td></td>
</tr>
<tr>
<td>Date test completed:</td>
<td></td>
</tr>
<tr>
<td>Mean diameter of lesions (for each dilution):</td>
<td></td>
</tr>
<tr>
<td>Result:</td>
<td></td>
</tr>
</tbody>
</table>

Production of culture medium (section A.3.3)

Any components of animal origin: ____________________________________________
Certificate for BSE/TSE-free: ______________________________________________
Control of vaccine production (section A.4)

Control of single harvests (section A.4.1)

Derived from master seed lot number: ________________________________
Working seed lot number: ________________________________
Passage level from master seed: ________________________________
Culture medium: ________________________________
Number and volume of containers inoculated: ________________________________
Date of inoculation: ________________________________
Temperature of incubation: ________________________________
Date of harvest: ________________________________
Results of visual inspection: ________________________________

Control of final bulk (section A.4.2)

Tests for bacterial and fungal contamination (section A.4.2.2)

Method used: ________________________________
Number of containers tested: ________________________________
Volume of inoculum per container: ________________________________
Volume of medium per container: ________________________________
Observation period (specification): ________________________________

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

Test for absence of virulent mycobacteria (section A.4.2.3)
(if not performed on final lot)

Method used: ________________________________
No. of human doses injected per guinea-pig: ________________________________
Inoculation route: ________________________________
No. of guinea-pigs given injection: ________________________________
Weight range of guinea-pigs: ________________________________
Observation period (specification): ________________________________
Date test started: ________________________________
Date test completed: ________________________________
Health of animals during test: ________________________________
Weight gains (losses): ________________________________
Result: ____________________________________________

Test for bacterial concentration (section A.4.2.4)
Method used: _______________________________________
Date test started: ____________________________
Date test completed: ____________________________
Specification: ____________________________________
Result: ____________________________

Test for number of culturable particles (section A.4.2.5)
Method used: _______________________________________
Date test started: ____________________________
Date test completed: ____________________________
Specification: ____________________________________
Result: ____________________________
Details of working Reference Preparation: _______________________

Substances added (section A.4.2.6)
Any components of animal origin: _______________________
Certificate for BSE/TSE-free: ___________________________

Filling and containers (section A.5)
Lot number: ____________________________
Date of filling: ____________________________
Volume of final bulk filled: ____________________________
Filling volume per container: ____________________________
Number of containers filled (gross): ____________________________
Date of freeze-drying: ____________________________
Number of containers rejected during inspection: ____________________________
Number of containers sampled: ____________________________
Total number of containers (net): ____________________________
Maximum period of storage approved: ____________________________
Storage temperature and period: ____________________________

Control tests on final lot (section A.6)
Inspection of final containers (section A.6.1)
Appearance: ____________________________
Date of test: ____________________________
Specification: ____________________________
Result: ________________________________
Recommended reconstitution fluid: ________________________________
Volume of reconstitution fluid per final container: ________________________________

Identity test (section A.6.2)
Method used: ________________________________
Date test started: ________________________________
Date test completed: ________________________________
Specification: ________________________________
Result: ________________________________

Tests for bacterial and fungal contamination (section A.6.3)
Method used: ________________________________
Number of containers tested: ________________________________
Volume of inoculum per container: ________________________________
Volume of medium per container: ________________________________
Observation period (specification): ________________________________
Specification: ________________________________

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

Safety tests (section A.6.4)
Test for absence of virulent mycobacteria (section A.6.4.1) (if not performed on final bulk)
Method used: ________________________________
No. of human doses injected per guinea-pig: ________________________________
Inoculation route: ________________________________
No. of guinea-pigs given injection: ________________________________
Weight range of guinea-pigs: ________________________________
Observation period (specification): ________________________________
Date test started: ________________________________
Date test completed: ________________________________
Health of animals during test: ________________________________
Weight gains (losses): ________________________________
WHO Expert Committee on Biological Standardization

Sixty-second report

Test for excessive dermal reactivity (section A.6.4.2) if applicable

<table>
<thead>
<tr>
<th></th>
<th>Vaccine</th>
<th>Reference vaccine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method used</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dilutions injected</td>
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<td></td>
</tr>
<tr>
<td>Inoculation route</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of guinea-pigs given injection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Observation period (specification)</td>
<td></td>
<td></td>
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<tr>
<td>Date test started</td>
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<tr>
<td>Date test completed</td>
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<td></td>
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<tr>
<td>Mean diameter of lesions (for each dilution)</td>
<td></td>
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</table>

Specification: ____________________________________________________________
Result: ________________________________________________________________

Test for bacterial concentration (section A.6.5)

<p>| | | |</p>
<table>
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<tr>
<td>Method used</td>
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<td>Date test completed</td>
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<tr>
<td>Specification</td>
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<tr>
<td>Result</td>
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</table>

Test for residual moisture (section A.6.6)

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<tr>
<td>Specification</td>
<td></td>
<td></td>
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<tr>
<td>Result</td>
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</table>

Tests for viability (section A.6.7)

Test for number of culturable particles (section A.6.7.1)

<table>
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<th>Before lyophilization</th>
<th>After lyophilization</th>
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</thead>
<tbody>
<tr>
<td>No. of containers tested</td>
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<td></td>
</tr>
<tr>
<td>Mean count of culturable particles per ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean survival rate (%)</td>
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<td></td>
</tr>
</tbody>
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<tbody>
<tr>
<td>Method used</td>
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<td></td>
</tr>
<tr>
<td>Medium</td>
<td></td>
<td></td>
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<tr>
<td>Date test started</td>
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<td></td>
</tr>
<tr>
<td>Date test completed</td>
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<th></th>
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<tbody>
<tr>
<td>No. of containers tested</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean count of culturable particles per ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean survival rate (%)</td>
<td></td>
<td></td>
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</tbody>
</table>
Annex 3

Specification: 
Result: 
Details of working Reference Preparation: 

Rapid test for viability (section A.6.7.2) if applicable
Method: 
Mean survival rate (%): 
Date: 
Specification: 
Result: 

Thermal stability test (section A.6.8)
Method used: 
Date test started: 
Date test completed: 

<table>
<thead>
<tr>
<th></th>
<th>Unheated containers</th>
<th>Heated containers</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of containers tested:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Culturable particles in each container per ml:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean survival rate (%):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specification:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Result:</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Details of working Reference Preparation: 

Submission addressed to NRA

Name of responsible person (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 BCG vaccine, whose number appears on the label of the final container, meets all national requirements and/ or satisfies Part A\(^1\) of the WHO Recommendations to assure the quality, safety and efficacy of BCG vaccines (2013)\(^2\)

Signature: 
Name (typed): 
Date: 

---

\(^1\) With the exception of provisions on distribution and shipping, which the NRA may not be in a position to assess.

Appendix 3

Model certificate for the release of BCG vaccine by NRAs

Lot release certificate

Certificate no. _______________________

The following lot(s) of BCG vaccine produced by ______________________1 in ____________________,2 whose numbers appear on the labels of the final containers, complies with the relevant specification in the marketing authorization and provisions for the release of biological products3 and Part A4 of the WHO Recommendations to assure the quality, safety and efficacy of BCG vaccines (2013)5 and comply with WHO good manufacturing practices: main principles for pharmaceutical products;6 Good manufacturing practices for biological products;7 and Guidelines for independent lot release of vaccines by regulatory authorities.8

The release decision is based on ____________________________9

The certificate may include the following information:

- name and address of manufacturer;
- site(s) of manufacturing;
- trade name and common name of product;
- marketing authorization number;
- lot number(s) (including sub-lot numbers, packaging lot numbers if necessary);

---

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 NRA.
4 With the exception of provisions on distribution and shipping, which the NRA may not be in a position to assess.
9 Evaluation of summary protocol, independent laboratory testing, and/or specific procedures laid down in defined document etc., as appropriate.
- type of container;
- number of doses per container;
- number of containers/lot size;
- date of start of period of validity (e.g. manufacturing date) and/or expiry date;
- storage condition;
- signature and function of the authorized person and authorized agent to issue the certificate;
- date of issue of certificate;
- certificate number.

The Director of the NRA (or other authority as appropriate):

Name (typed) ____________________________________________________________
Signature _____________________________________________________________
Date _________________________________________________________________
Annex 4

Recommendations to assure the quality, safety and efficacy of acellular pertussis vaccines


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Recommendations published by WHO are intended to be scientific and advisory in nature. The parts of each section printed in type of normal size have been written in such a form that, should a national regulatory authority (NRA) desire, they may be adopted as they stand as definitive national requirements or used as the basis of such requirements. Those parts of each section printed in small type are comments and additional guidance. It is recommended that modifications 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.
Introduction

Pertussis immunization is an integral part of immunization programmes in all regions of the world. It is recommended for all infants and children and in some countries it is also recommended for adults and adolescents. Whole-cell pertussis vaccines, which have been used for more than 50 years, have been shown to provide protection against pertussis and still serve as the foundation of global pertussis control. However, there is an increasing interest in acellular pertussis vaccines which have also been shown to be safe and effective and which have been successfully introduced into many national immunization programmes. A detailed comparison of acellular and whole-cell pertussis vaccines is beyond the scope of this document; however, these issues are discussed in detail in a WHO position paper on pertussis vaccines (1). As a consequence of the increasing demand for acellular pertussis vaccines, new manufacturers are entering the field. The expansion in the number and use of acellular pertussis vaccines, the development of new vaccines and advances in the standardization of quality-control methods have prompted WHO to update its current Guidelines for acellular pertussis vaccines (2).

These Guidelines were approved in 1996, with the recognition that further improvements in the production and evaluation of these vaccines would follow. Since then, stakeholders have gained additional experience with these vaccines, and limitations in the original Guidelines have been identified (3–7). Acellular pertussis vaccines are almost exclusively administered in combinations with diphtheria and tetanus toxoid vaccines. Moreover, in recent years there has been increased interest in the use of more complex combination vaccines – a trend which increases the challenges of clinical evaluation. Furthermore, the evaluation of the clinical efficacy of any new acellular pertussis vaccine formulations has become increasingly difficult due to the decrease in the prevalence of pertussis cases worldwide and for additional reasons discussed below in Part C. The goal of this revision is to address these issues concerning the Guidelines in the light of new information.

Background

The development of acellular pertussis vaccines was stimulated by scientific advances that led to the identification of components such as toxins and surface proteins of Bordetella pertussis that are believed to play a role in pathogenesis and induction of protective immunity. The first acellular pertussis vaccines were produced through a purification process that resulted in the enrichment of two protein components – namely pertussis toxin (PT) and filamentous haemagglutinin (FHA) – that were protective in animal models, and were introduced for routine use in Japan around 1980 as a response to increasing public concern over adverse
reactions to whole-cell pertussis vaccines. These vaccines were prepared from cell-free *B. pertussis* culture supernatants by ultracentrifugation, and all contained FHA and PT that were treated with formaldehyde to inactivate the PT. These vaccines also contained various amounts of other *B. pertussis* proteins as minor components, including pertactin (PRN or 69 kDa protein) and fimbriae (FIM). Epidemiological evaluations indicated that these vaccines effectively controlled pertussis disease when introduced for routine immunization (8–10).

An alternative approach to manufacturing employed individually purified pertussis antigens which, after detoxification, were used to formulate vaccines of defined composition. These purified component vaccines varied in the number of antigens incorporated, and in the PT detoxification procedures and antigen-purification processes used.

Between 1986 and 1996, several vaccines containing acellular pertussis, including some composed of purified antigens and some composed of co-purified antigens, were evaluated in a series of efficacy trials. These trials evaluated acellular pertussis vaccines containing up to five pertussis components and utilized different study designs, including randomized placebo-controlled cohort trials, household contact studies, and case–control studies (11, 12). This series of trials revealed that all the tested acellular pertussis vaccines provided some protection against pertussis, although the studies suggested differences between the vaccines. Additional detail is provided in Part C of these Recommendations. Unless vaccines of different types were tested in parallel within the same trial, comparing efficacy among different acellular vaccines must be done with caution as all the trials varied with respect to design, case-ascertainment methodology and case definition. Following the completion of these trials, many countries introduced acellular pertussis vaccines into their routine immunization programmes.

In addition to heterogeneity in production and composition, there are variations in the approaches used for control testing of acellular pertussis vaccines. The testing methodology developed in Japan was based on modifications of the tests used for whole-cell vaccines. This included a modification of the mouse intracerebral challenge assay for potency, along with additional requirements designed to monitor purity, content and residual toxin activities. In general, these tests and specifications are not vaccine-specific and can be used for evaluating new products with new antigen formulations. Alternative control testing approaches were adopted initially in Europe and North America for purified component vaccines based on the concept that the newly manufactured lots should be comparable to those evaluated in pivotal clinical studies. Control testing of these vaccines included in-process and final product tests for purity, composition, residual toxin activity and immunogenicity. As the products differ markedly from each other, specifications were product-specific and were based on the concept that the newly manufactured lots should closely match those evaluated in clinical studies. The immunogenicity of individual antigens
using antibody-binding assays was used as one of the markers of production consistency. The different methods used to evaluate potency and residual toxin activity are designed to assess different characteristics of the vaccines. However, acellular pertussis vaccines tested by both approaches have been used effectively. Importantly, both testing approaches have been applied to purified component vaccines and to co-purified vaccines. Further research is needed to develop alternative methods and to standardize the current potency and safety tests used for the evaluation of acellular pertussis vaccines.

**Scope**

These Recommendations apply to co-purified and purified component acellular pertussis vaccines. The document covers only antigens produced by *B. pertussis*. While other approaches are possible (e.g. antigens produced from *B. bronchiseptica* or *Escherichia coli*) they are not considered in this document.

Although these Recommendations apply to the production and quality control of acellular pertussis vaccines, the acellular pertussis component is combined most commonly with other antigens (e.g. diphtheria and tetanus toxoids, *Haemophilus influenzae* type b conjugate vaccine, inactivated polio vaccine and hepatitis B). Therefore the tests recommended for the final bulk or final product of acellular pertussis vaccines should be performed on the final bulk or final product of the combined vaccines.

These revised Recommendations highlight the advances made in the development, manufacturing and testing of acellular pertussis vaccines and aim to provide guidance on the following issues:

- improvement of quality control of existing vaccines on the basis of new information and experience;
- evaluation of new products and new combinations through control of manufacturing;
- evaluation of the vaccines in both nonclinical and clinical studies.

The main changes made to the WHO Guidelines published in 1998 are as follows.

- The title of the document is upgraded from “Guidelines” to “Recommendations”.
- Advice on clinical and nonclinical evaluation of acellular pertussis vaccines is added to guide NRAs and vaccine manufacturers in approaches that can be used to assess the safety, efficacy and quality of vaccines.
Details are provided for the modified intracerebral challenge assay used to evaluate the potency of the acellular pertussis vaccine (Appendix 1), and for the histamine sensitization test (HIST) based on temperature measurement to determine the residual activity of PT in the vaccine (Appendix 2).

Information is provided for the performance of the mouse respiratory challenge method. Although the method is currently not recommended for routine potency testing, it may have an important role in nonclinical testing as discussed in Part B of these Recommendations.

General considerations

Written descriptions of detailed procedures or the standard operating procedures used for the production and testing of the acellular pertussis vaccines or combined vaccines containing acellular pertussis component(s), together with evidence of appropriate validation of each production step and relevant control tests, should be submitted for approval to the NRA as part of the licence application. Proposals for any variations in manufacturing and/or control methods should be submitted for approval to the NRA according to national regulatory requirements before they are implemented.

There is as yet no consensus on the ideal antigenic composition of acellular pertussis vaccines. Currently, various acellular pertussis vaccine products are available from diverse manufacturers, differing in the number of components, their concentrations and their degree of adsorption to different adjuvants. In addition, these individual antigens have been derived from different strains of *B. pertussis*, purified by different methods and treated with different detoxification agents. All currently available acellular pertussis vaccines contain detoxified PT (PTxd) and some vaccines formulated with PTxd alone have been shown to provide a significant degree of protection. However, clinical and laboratory studies have suggested that the protective efficacy of PT may be enhanced by other antigens (11–19). Ongoing research is essential to identify the protective mechanisms, to identify immunological markers of protection against pertussis, and to develop and improve relevant laboratory models. Additionally, because all current acellular pertussis vaccines are administered in more complex combination vaccines, research is encouraged on models that allow the concurrent testing of multiple components (e.g. diphtheria and tetanus toxoids).

Manufacturers should demonstrate consistency in manufacturing and formulation and should adhere strictly to the production process used for the manufacture of the vaccine lots used in the clinical trials supporting regulatory approval. In addition, laboratory tests should show consistency in the safety,
Annex 4

potency, and physicochemical and immunological characteristics of new vaccine lots compared with lots evaluated in clinical studies. Special care should be taken in the validation of those test procedures that are used for ensuring consistency of production lots for specificity, sensitivity and accuracy. Manufacturers should ensure that sufficient quantities of reference vaccine of adequate stability are available for routine in-house testing and for confirmatory tests undertaken by the NRA.

An in-house standard, when used, should be assigned a value so that trend analyses can be monitored and quality-control testing limits can be defined. When an International Standard exists, the in-house standard should be calibrated against that standard.

Use of a suitable freeze-dried vaccine preparation as an in-house reference may offer advantages of stability. A successful example of conditions which have been used for diphtheria vaccines (adsorbed) is as follows: vaccine lyophilized in the presence of 3.5% polygeline (1:1) under freeze-drying cycle at −50 °C load, −50 °C freeze over 2.5 hours, then primary drying at −35 °C (100 µbar vacuum) and secondary drying at 30 °C (30 µbar vacuum) (20).

There are no laboratory tests, animal models and/or human immune responses that can provide complete assurance that a newly developed acellular pertussis vaccine will be adequately safe and effective. Within these limitations, these Recommendations describe a sequential approach to the collection of supporting evidence, beginning with a comprehensive programme of nonclinical testing and followed by a progression of clinical evaluations. For the purpose of this document, a newly developed vaccine would be any vaccine that contains either a novel antigen or one of the antigens in the currently licensed products (i.e. PTxd, FHA, PRN, FIM type 2 and FIM type 3) that is produced from a new strain, new process and/or new manufacturer. As described in Part B, nonclinical characterization studies should include evaluations of purity, residual toxin activity, bioactivity, reactivity with specific antibodies, induction of binding and functional antibodies, and induction of protective activity in animal models. Whenever an additional antigen is added, studies should be undertaken to characterize its interaction with other components in the product. If the antigen is novel, more extensive characterization studies would be expected. This document assumes that only those vaccines that have already undergone extensive nonclinical testing would be considered for clinical evaluation, with agreement with the local NRA responsible for evaluating adequacy of nonclinical information. Although efficacy trials appear very difficult, if not impossible, safety and immunogenicity trials of adequate design and size are possible and should be conducted. In Part C, the Recommendations provide guidance on issues related to the design and
evaluation of the clinical studies. Most studies are expected to be comparative studies. Thus the choice of a comparator vaccine is a particularly important issue because the potential comparator vaccines differ substantially in formulation and composition. Finally, because the tools for clinical evaluation are limited, rigorous post-marketing monitoring of the vaccines will be needed in order to determine if they are achieving acceptable levels of clinical safety and efficacy.

Given these limitations, some caution is appropriate when considering a transition from whole-cell to acellular pertussis vaccines. Specifically, whole-cell pertussis vaccines are safe and effective, and offer some advantages as described in the WHO position paper (1). Thus, although these Recommendations describe an approach for approval of new acellular pertussis vaccines, the path for approval of new acellular pertussis vaccines requires considerable effort and includes significant challenges.

**International Standards and Reference Preparations**

Subsequent sections of this document refer to WHO reference materials that may be used in laboratory or clinical evaluations. The WHO catalogue of international biological standards should be consulted for the latest list of appropriate WHO International Standards and reference materials (see: [http://www.who.int/bloodproducts/catalogue/en/index.html](http://www.who.int/bloodproducts/catalogue/en/index.html)). Key standards and reference materials include:

- the First WHO International Standard for acellular pertussis vaccine for use in modified mouse intracerebral challenge assay (MICA) and other protection assays (Code no. JNIH 3 with assigned unitage per ampoule of 34 IU);
- the First WHO International Standard for pertussis toxin for standardization of assays used to monitor the residual PT activity in pertussis vaccines, e.g. histamine sensitization tests and Chinese hamster ovary (CHO) cell assay (Code no. JNIH 5 with assigned unitage per ampoule of 10 000 IU);
- the First WHO International Standard for pertussis antiserum (human) (Code no. 06/140 with assigned unitage per ampoule of 335 IU anti-PT IgG and 65 IU IgA anti-PT; 130 IU IgG anti-FHA and 65 IU IgA anti-FHA; 65 IU IgG anti-69K and 42 IU IgA anti-69K);
- the First WHO International Reference Reagent for standardization of clinical serology assays (Code no.06/142 with assigned unitage per ampoule of 106 IU anti-PT IgG and 18 IU IgA anti-PT; 122 IU IgG anti-FHA and 86 IU IgA anti-FHA; 39 IU IgG anti-69K and 38 IU IgA anti-69K);
■ the First WHO International Standard for monoclonal antibody to *B. pertussis* fimbriae type 2 for the determination of serotype of *B. pertussis* strains (Code no. 06/124);

■ the First WHO International Standard for monoclonal antibody to *B. pertussis* fimbriae type 3 for the determination of serotype of *B. pertussis* strains (Code no. 06/128);

■ the First WHO International Reference Reagent for pertussis antiserum (mouse) (Code no. 97/642 with assigned unitage per vial of 17 units of anti-PT, 143 units of anti-FHA, 30 units of anti-PRN and 32 units of anti-FIM 2 and 3).

The above-mentioned WHO International Standards and reference materials and other reagents from the WHO International Standards Laboratory are in the custody of the National Institute for Biological Standards and Control (NIBSC), Health Protection Agency, Potters Bar, England (see: http://www.nibsc.ac.uk).

These Reference Preparations are available for the calibration and establishment of regional, national or in-house reference materials. Samples are distributed free of charge, on request, to NCLs.

### Terminology

The definitions given below apply to some common terminology used throughout this document. The terms may have different meanings in other contexts.

**Master seed lot**: a quantity of bacterial suspension that is derived from a single strain, has been processed as a single lot and has a uniform composition. It is used for inoculating media for the preparation of working seed lot.

**Working seed lot**: a quantity of bacterial suspension of a single substrain derived from the master seed lot by growing the organisms and maintaining them in aliquots in the frozen form or in the lyophilized form, stored at –20 °C or below (in the liquid form stored at –80 °C or below). The working seed lot should be prepared from the master seed lot by as few cultural passages as possible, having the same characteristics as the master seed lot and intended for inoculating media for the preparation of single harvests.

**Single harvest**: the culture filtrate or the suspension of bacteria obtained from one batch of cultures that have been inoculated with the working seed lot (or with the inoculums derived from it), harvested and processed together.

**Purified antigen(s) bulk material**: the processed purified material prepared using pertussis antigen preparations processed either in a single run or a pool of those prepared in multiple runs. In some cases, purified antigen bulk
material may be adsorbed to or mixed with adjuvant and a preservative may be added. It is the parent material from which the final bulk is prepared.

**Final bulk:** the homogeneous finished vaccine present in a single container from which the final containers are filled either directly or through one or more intermediate containers.

**Final lot or final product:** 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.

**Individually purified antigen:** each of the pertussis antigens that are individually isolated and purified using combinations of several physicochemical separation methods.

**Co-purified antigen:** two or more pertussis antigens that have been isolated and purified using combinations of several physicochemical separation methods (e.g. ammonium sulfate precipitation and density gradient centrifugation).

**Comparator vaccine:** an approved vaccine with established efficacy or with traceability to a vaccine with established efficacy that is tested in parallel with an experimental vaccine and serves as an active control in nonclinical or clinical testing.

### Part A. Manufacturing recommendations

**A.1 Definitions**

**A.1.1 International name and proper name**

The international name should be acellular 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**

Acellular pertussis vaccine is a preparation of purified or co-purified antigenic component(s) of *Bordetella pertussis* that have been appropriately treated by chemical means or obtained by genetic manipulation to minimize toxicity and retain potency. The preparations for human use should satisfy all the recommendations formulated below.

Currently licensed vaccines contain either PTxd alone or PTxd in combination with one or more other antigens (FHA, PRN, fimbriae type 2 [FIM-2] and fimbriae type 3 [FIM-3]).
A.2 **General manufacturing recommendations**

The general manufacturing requirements contained in good manufacturing practices (GMP) for pharmaceutical (21) and biological products (22) apply to the production of the acellular pertussis vaccines.

A.3 **Production control**

These Recommendations pertain to antigen production and purification from *B. pertussis*.

A.3.1 **Control of source materials**

A.3.1.1 **Strains of *Bordetella pertussis***

Strains of *B. pertussis* used in preparing vaccine should be identified by a full record of their history, including origin and characteristics on isolation, and particulars of all tests made periodically to verify strain characteristics. If genetically modified *B. pertussis* is used, all relevant modified DNA sequences should be clearly delineated and fully characterized. The strains of *B. pertussis* used should be approved by the NRA.

A.3.1.2 **Seed lot system**

The production of the acellular pertussis component of monovalent or combined vaccines should be based on a well-established seed lot system. Cultures from the working seed lot should have the same characteristics as cultures from the master seed lot. If genetically modified *B. pertussis* is used, the relevant modified DNA sequences should be reconfirmed for each new working seed lot. The strains should be maintained by a method approved by the NRA and able to preserve the ability of the seed to yield potent vaccine in terms of the quality of the antigens produced.

Freeze-drying or storage in liquid nitrogen are satisfactory methods of maintaining strains, subject to suitable validation. In some countries, glycerol stocks are also used for this purpose, but this method would require extensive validation and approval of the NRA.

A.3.1.3 **Culture media for production**

*B. pertussis* should be cultured in culture media suitable to support its growth and the production of relevant antigens with consistent yields. Media used should be free from adventitious agents. Moreover, medium components that are known to cause allergic reactions should be avoided. Human blood or blood products should not be used in culture media either for seed lots or for vaccine production. The acceptability of the source(s) of any components of bovine, sheep or goat origin used in culture media should be approved by the NRA.
If any materials of animal origin are used in seed preparation or preservation, or in production, they should comply with the WHO guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products (23) and national regulatory requirements. When animal blood or blood products are used, they should be removed in the process of production by appropriate methods.

Any change of media should be submitted for approval to the NRA.

A.3.2 Control of manufacturing process

A.3.2.1 Control of production cultures

Production cultures should be shown to be consistent with respect to growth rate, rate of change of pH, rate of production of the desired antigen(s), and additional parameters as agreed with the NRA. Acceptance specifications should be established and agreed with the NRA.

A.3.2.2 Control of bacterial purity

Samples of individual cultures should be tested for microbial purity by microscopic examination of stained smears, by inoculation of appropriate culture media, or by any other suitable procedure. For microscopic examination, several fields should be examined at high magnification. If a contaminant is found, the culture and any product derived from it should be discarded.

In some countries the individual cultures are tested for microbial purity by a minimum of two suitable and approved procedures.

A.3.2.3 Control of antigen purification

Cultures should be processed for further antigen purification in a way that minimizes contamination of crude materials with undesirable molecules, such as lipooligosaccharide (LOS), dermonecrotic toxin, adenylate cyclase toxin (ACT), and tracheal cytotoxin (TCT). Cells of *B. pertussis* may be separated from fermentation fluid by filtration or centrifugation, and should be suitably inactivated before their further processing or disposal. Absence of cells of *B. pertussis* from crude antigen solutions should be confirmed at this stage using appropriate methods approved by the NRA.

Two approaches have been followed for the purification of pertussis antigens for vaccine manufacturing. In the first approach, antigens have been co-purified by repeated cycles of ammonium sulfate precipitation and density gradient centrifugation to yield preparations enriched in certain proteins – mainly PT, FHA and PRN – but depleted of endotoxin (LOS). The number and proportion of each antigen in a given vaccine type may vary widely depending on the process followed, but should be reproducible for each specific product.
In the second approach each antigen is individually purified using combinations of several physicochemical separation methods.

The tests used for determination of consistency of yield and purity and their performance characteristics should be approved by the NRA.

It is advisable to sterilize purified antigens by membrane filtration or other suitable sterilizing grade filtration before further processing.

A.3.2.3.1 Tests undertaken prior to detoxification/chemical treatment

**Characterization of antigens:** rigorous characterization of the antigens by physicochemical, immunological or functional (biological) assays, as appropriate, is essential before any step is undertaken that is capable of modifying their original characteristics. Particular attention should be given to employing a range of analytical techniques based on different principles. Immunoblot analysis with specific monoclonal or polyclonal antibodies may be used to characterize antigens or antigen subunits. The specific properties of each antigen component should be determined in comparison with Reference Preparations. Specifications should be established for each individual antigen. Suitable assays include sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), single radial immunodiffusion, immunoblotting, the CHO cell test for detection of active PT, haemagglutination and high-performance liquid chromatography (HPLC). The specific activity of PT (IU/ng) should be determined.

**Antigen purity:** the purity of the individual or co-purified antigens claimed to contribute to vaccine efficacy should be determined by SDS-PAGE, HPLC or other appropriate analyses before detoxification. It is important that the techniques used are based on as wide a range of properties of the vaccine components as possible. Limits should be specified for all impurities detected.

The purity of the individual or co-purified antigens should be within the range of values established for each product as found for vaccine lots shown to be safe and effective in clinical trials or other lots used in support of licensing. Specifications should be set during the process of product development and validation and should be established by agreement with the NRA.

**Residual levels of endotoxin:** the antigens should be tested for residual endotoxin content by means of the *Limulus* amoebocyte lysate (LAL) test or other appropriate assay. This test may be carried out at a later stage. Endotoxin content should be consistent with levels found in vaccine lots shown to be safe and effective in clinical trials or for lots used in support of licensing. Specifications should be established in agreement with the NRA.

**Antigen content:** if it is necessary at this stage, the amount of individually purified antigens that have been characterized and purified, as appropriate, should be estimated by means of a validated quantitative assay of sufficient sensitivity,
such as an assay for protein content and, where available, a suitable quantitative immunoassay. Antigen content can be determined by ELISA; active PT content can be determined by CHO cell assay.

In cases where two or more antigens are co-purified, the proportion of each antigen claimed to contribute to vaccine efficacy should be measured by a suitable method (e.g. SDS-PAGE, HPLC, electrophoresis on non-denaturing gels, or densitometry) and should be shown to be within the range of values found for vaccine lots shown to be safe and effective in clinical trials or other lots used in support of licensing. Specifications should be set during the validation and established in agreement with the NRA.

**Sterility test:** bacterial and mycotic sterility for each antigen lot should be determined in accordance with the requirements of Part A, section 5, of the revised Requirements for biological substances, No. 6 (General requirements for the sterility of biological substances) (24), or by a method approved by the NRA. If appropriate, this test may be carried out at a later stage.

If a preservative is added, appropriate measures should be taken to prevent interference with the sterility test.

### A.3.2.4 Detoxification

The purified PT, if it is not genetically detoxified, or co-purified antigens that contain this toxin should be subjected to appropriate detoxification methods. Other antigens may also be treated with agents to detoxify any residual PT in the preparation. The residual detoxifying agents should be removed by an appropriate method. Different chemicals are used to detoxify PT. These include, but are not limited to, formaldehyde, glutaraldehyde, a combination of both, or hydrogen peroxide. Different detoxification processes yield distinct products.

The detoxification method/process should be validated for the ability to consistently produce antigens that have acceptably low levels of biologically active PT and retain acceptable levels of immunogenicity, as measured on final formulation. In addition, the detoxification method should be validated for the ability to produce detoxified PT that does not revert to biologically active PT upon storage. If any aggregation of antigens has occurred following detoxification, the aggregates should be homogenized by an appropriate procedure such as sonication followed by filtration to remove the larger clumps.

### A.3.3 Control of pertussis antigen bulk materials

Bulk materials should be prepared using antigen preparations processed either in a single run or in a pool of those prepared in multiple runs. With the approval of the NRA, the bulk materials may be adsorbed to/mixed with adjuvant and a preservative may be added.
Antigen content: the amount of each individual antigen or of co-purified antigens should be estimated by means of a validated quantitative assay, such as an assay for protein content and, where available, suitable quantitative immunoassays for individual antigens. For co-purified antigens, the ratio of antigens should be defined. When no adequate procedure is available for assessing individual antigens following chemical detoxification, a validated suitable procedure may be used to estimate the amount of individual antigens based on the amounts measured before detoxification. Specifications for antigen content should be set during the process of product development and validation and should be approved by the NRA.

Residual activity of pertussis toxin: the amount of residual biologically active PT in the individually or co-purified antigens should be estimated after detoxification by means of a sufficiently sensitive test such as the HIST or the CHO cell assay. Adjuvants and other vaccine components may interfere with the adequate performance of the CHO cell assay (25) and special care must be taken to ensure that they do not interfere with the tests (e.g. by adequate dilution of test solutions). At the concentration of vaccine final formulation, the total amount of residual bioactive PT from all pertussis antigens should not exceed that found in vaccine lots shown to be safe in clinical trials or other lots used in support of licensing. Specification should be established in agreement with the NRA.

Residual levels of endotoxin: the bulk material or antigens should be tested for residual endotoxin content by means of the LAL test or other appropriate assay. At the concentration of vaccine final formulation, the total amount of residual endotoxin should not exceed that found in vaccine lots shown to be safe in clinical trials or other lots used in support of licensing. The limits applied to the vaccine concentration of individual components should be agreed with the NRA.

Sterility test: each purified antigen bulk should be tested for bacterial and mycotic sterility in accordance with the requirements of Part A, section 5, of the revised Requirements for biological substances, No. 6 (General requirements for the sterility of biological substances) (24), or by a method approved by the NRA. If a preservative is added, appropriate measures should be taken to prevent interference with the sterility test.

A.3.4 Control of final bulk
Currently there is no stand-alone acellular pertussis vaccine, so the tests described below are undertaken at the final bulk stage of combined vaccines.

If a stand-alone acellular pertussis vaccine were to be developed, the procedures described here could be adapted for such a product.
A.3.4.1 Preparation

The antigen bulk materials should be pooled to prepare the pertussis bulk. Current preparations may contain PTxd alone or together with FHA, with or without PRN and FIM 2 and 3, to produce one, two, three, four or five component acellular pertussis vaccines. The bulk of acellular pertussis vaccine may be adsorbed to/mixed with aluminium hydroxide or phosphate gel or another appropriate adjuvant prior to or after blending with other components (e.g. diphtheria toxoid, tetanus toxoid, inactivated polio vaccine (IPV)) to produce the final formulation (final bulk). A suitable antimicrobial preservative may be added.

A.3.4.2 Control tests

The following control tests on final bulk may be performed on the final product in agreement with the NRA.

A.3.4.2.1 Detoxifying agents

The content of residual detoxifying agent in the final bulk should be determined. The method and limits should be approved by the NRA.

If formaldehyde has been used, the residual content should not exceed 0.2 g/l. The residual content of glutaraldehyde should not exceed 0.1 g/l.

A.3.4.2.2 Preservative

Consideration should be given to the effect of the preservative on the stability of the vaccine formulation and possible interactions between the vaccine components and the preservative. The content of preservative should be determined by a method approved by the NRA. The amount of preservative in the vaccine dose should be shown to have no deleterious effect on the antigen(s) and should not impair the safety of the product for humans. The preservative, its use at different stages of the manufacturing process, and its concentration or residual amount should be approved by the NRA. If any modification of preservative content in 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 (26).

Phenol should not be used as a preservative.

A.3.4.2.3 Adjuvant

The nature, purity and concentration of the adjuvant or adjuvants added to the vaccine should be determined by a method approved by the NRA. When aluminium compounds are used as adjuvant the concentration of aluminium should not exceed 1.25 mg per single human dose. When calcium adjuvants
are used, calcium should not exceed 1.3 mg per single human dose. If other substances are used as adjuvants, specifications should be set and agreed with the NRA. The formulation should be such that the suspension appears homogeneous after shaking and remains as such for a defined period (e.g. the time needed for vaccine administration). Adsorption of antigens to the adjuvant should be investigated, when possible, by tests designed to determine which, and how much of each, are adsorbed. Consistency of adsorption is important, and the adsorption of the antigen in production lots should be demonstrated to be within the range of values found for vaccine lots which have been shown to be clinically safe and effective, or for lots used in support of licensing.

A.3.4.2.4 Sterility

Each final bulk should 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) (24) or by a method approved by the NRA.

If a preservative has been added to the vaccine, appropriate measures should be taken to prevent it from interfering with the test.

A.3.4.2.5 Residual activity of pertussis toxin

Each final bulk of vaccine should be tested for active PT using a HIST or another test that is sufficiently sensitive to detect the level of toxin activity agreed with the NRA. For products containing genetically detoxified PT, this test may not be necessary if agreed with the NRA. Currently the HIST method used for the determination of residual bioactive PT in licensed acellular pertussis vaccines is based on the response to a histamine challenge of mice injected with the test vaccine. Two possible test outcomes (end-points) are in use. One is based on the lethal effect of the histamine challenge dose and the other on the decrease in body temperature following the histamine challenge. If an alternative assay is used, it should be at least as sensitive and specific as a validated HIST assay (either of the two tests mentioned above). The alternative method(s) should be approved by the NRA.

The susceptibility to histamine sensitization of the mice used in each test should be established using a suitable reference or control preparation of PT. The specific activity of the standard or positive control should be calibrated using the WHO International Standard (currently JNIH-5) and should be expressed in IU because the nominal protein masses of the toxin preparations do not necessarily predict their biological activity in HIST. The detection limit for the PT of the test should be defined and accepted by the NRA.

The acceptance criteria for content of residual bioactive PT should be consistent with the results for lots shown to be safe and effective in clinical trials.
or other lots used in support of licensing. Specifications should be established in agreement with the NRA.

There is at present limited information about the relation between the level of residual active PT in an acellular pertussis vaccine and its clinical safety. Therefore the residual toxicity should be reduced as far as is feasible without undue compromise of immunogenicity. There is no internationally agreed upper limit for active PT in acellular pertussis vaccines. In some countries, upper limits of PT per single vaccine dose are a requirement for DTaP vaccines (27, 28). A recent collaborative study has provided preliminary data on the content of bioactive PT in DTaP-based combination vaccines using the HIST lethal end-point method (29).

The HIST lethal end-point method (see Appendix 3) measures the proportion of animals that die upon histamine challenge due to sensitization with residual PT in the vaccine. Assay sensitivity is verified by titration of a PT standard (calibrated in IU). Once linearity has been established by repeated experiments, the assay may be simplified to include in each test only a single dose of PT standard to ensure assay sensitivity.

Some laboratories include a standard toxin at a concentration near the acceptance limit to verify assay sensitivity.

The HIST based on measurement of the reduction in temperature (either rectal or dermal) produced by histamine challenge (see Appendix 2) has been successfully used in some countries. It has also been optimized to provide a quantitative estimate of the activity of a test vaccine relative to the activity of a PT standard.

Although the CHO cell assay is highly sensitive for detection of PT activity, the test may not be suitable for the final bulk vaccine because of possible interference (e.g. presence of adjuvant or inactivating agents).

Development of an alternative to HIST is encouraged. An in vitro assay system comprising an enzymatic HPLC (E-HPLC) assay and a carbohydrate binding assay is under evaluation as a potential alternative to the HIST (30). Any alternative assay to the current HIST to determine the residual PT activity should be validated and approved by the NRA.

A.3.4.2.6 Reversion to toxicity

Accelerated reversion testing, consisting of HIST performance on final bulk or the final lot incubated for at least four weeks at 37 °C, may be used to demonstrate that it is unlikely that the chemically inactivated PT will regain some of its toxicity before the vaccine expiry date. Some NRAs may not require this test for the release of each new lot but only as part of process validation. For products
containing genetically detoxified PT, this test may not be necessary as agreed with the NRA.

A.3.4.2.7 Immunological activity

Two methods are currently used in the lot release procedure to assess the immunogenicity or potency in mice after vaccination with acellular pertussis vaccine: the mouse immunogenicity test (MIT) and the MICA. Importantly, none of these assays can be considered as an index of clinical efficacy. Use of any of these tests requires validation and agreement with the NRA. Active PT in the vaccine (if any) may enhance the potency of vaccines obtained in these assays, with the degree of enhancement depending on the immunizing antigens, mouse strain, test method and assay conditions (31, 32).

The MIT is a non-lethal animal model designed to evaluate antibody responses in immunized mice to all the antigens claimed to contribute to vaccine efficacy. Currently, ELISA is used to measure the binding activity rather than the functional activity of antibody for each of the antigens. An international mouse reference serum containing antibodies to five antigens is available to monitor consistency of the MIT stage.

The MIT is designed to assess consistency of manufacture by evaluating whether the results of the MIT for lots manufactured post-licensure are consistent with the results of lots with acceptable performance in clinical trials. Due to the different composition of different vaccine products, specifications are product-specific. Additionally, to ensure adequate performance, MIT requires the use of a product-specific reference or control vaccine analogous in composition to the test vaccine.

In the absence of an international reference vaccine for MIT, each manufacturer is responsible for the development of a reference vaccine that can allow for meaningful assessment of the immunogenic activity of the test vaccine with respect to the established specifications. Although a clinical lot with established efficacy may be considered as the reference vaccine, this is usually impractical due to considerations such as availability and long-term stability. However, the vaccine lot selected as reference should be sufficiently similar to the clinical lots in composition, manufacturing process, immunogenicity and/or protective effect. Stabilization of the reference vaccine is recommended, but careful attention should be given to any effects the stabilizing procedure may have on its activity.

The manufacturer is responsible for monitoring the stability of the reference and for replacement as needed. When monitoring stability of the reference or testing a candidate replacement for the reference, testing approaches that allow for higher precision (e.g. more tests, more animals per test, or more dilutions per test) are encouraged in order to improve the ability to detect changes in activity.
Establishment of the specifications for each product should be based on the response observed in the test for vaccine clinical lots and other lots used in support of licensing. The specifications for the antibody response to each antigen claimed to contribute to efficacy should be established and approved by the NRA. Additional details on the method are provided in Appendix 4.

Tests which show only that the test product does not differ significantly from the reference vaccine are not recommended. Specifications must be carefully justified and should take into account the precision of the test and the maximum allowable deviation from the reference vaccine.

The MICA: is a lethal challenge model in mice which detects mouse protective activity provided by the vaccine. The potency of each final bulk is expressed as a relative potency to a reference vaccine. That reference vaccine should be calibrated against the WHO International Standard for acellular pertussis vaccines (currently JNIH-3) (33) and the protective activity should be expressed in IU.

The assay method and the specifications should be approved by the NRA. Additional details on the method are provided in Appendix 1.

The specifications, where used currently, are that the vaccine passes the potency test if the result of a statistically valid assay 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 for primary immunization vaccine.

Active PT has been shown to enhance the relative potency of vaccines obtained in this assay, with the degree of enhancement depending on mouse strain, assay conditions etc. (32).

Other assays
The development of alternative assays to MIT and MICA is encouraged.

An alternative assay – the guinea-pig immunogenicity test – has been adopted in some countries (34, 35). The assay allows immunogenicity testing of the acellular pertussis components and the diphtheria and tetanus toxoid components using the same group of animals. Adoption of this or another alternative method for routine lot release would require validation and approval by the NRA.

Respiratory challenge method(s) such as the mouse intranasal challenge assay (INCA) (see Appendix 5) have been evaluated in WHO international collaborative studies. They have discriminated between vaccines with different protective capacity in mice. The current respiratory challenge assays are not optimized or designed for use as a routine test for determining vaccine potency. Nevertheless, they can be used to assess potential impact of changing the manufacturing process and/or
formulation, the activity and stability of new antigens and formulations, or the potential interactions in new combinations. This model is an important tool in new product development as described in Part B of this document.

A.4 **Filling and containers**
The requirements concerning filling and containers given in Good manufacturing practices for biological products (22) should apply to vaccine filled in the final form.
Single dose and multiple dose containers may be used. Vaccine in multidose containers should contain a suitable antimicrobial preservative.

A.5 **Control of final product**
Quality control procedures and tests should be validated and approved by the NRA to ensure that the final containers contain the appropriate amounts of each of the vaccine antigens, as designed for the acellular vaccine formulation.

Unless otherwise justified and authorized, the following tests should be performed on labelled containers from each final lot by means of validated methods approved by the NRA.

A.5.1 **Identity**
An identity test should be performed on at least one container from each final lot by means of a validated method approved by the NRA.

A.5.2 **Sterility**
Final containers should be tested for sterility by a method approved by the NRA. 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 (24). If a preservative has been added to the vaccine, appropriate measures should be taken to prevent it from interfering with the test.

A.5.3 **Adjuvant content**
The content of adjuvant should be determined by a method approved by the NRA. 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 are used as adjuvants, appropriate specifications should be set for the substance with adjuvant effect.

A.5.4 **Preservative content**
The content of preservative(s) should be determined by methods approved by the NRA. The amount of preservative per dose should be shown not to have
any deleterious effect on the antigen(s) nor cause untoward adverse reactions in humans. The preservative and its concentration or residual amount should be approved by the NRA. 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 (26).

A.5.5 pH
The pH of each final lot should be within the range of values found for vaccine lots shown to be clinically safe and effective.

In some countries, determinations for osmolality and withdrawable content are also required.

A.5.6 Endotoxin
In some countries, determination of endotoxin content may be required with specifications approved by the NRA.

A.5.7 Innocuity test
Each final lot should be tested for unexpected toxicity (sometimes called abnormal toxicity or innocuity) by a method approved by the NRA.

This test may be omitted for routine lot release once consistency of production has been well established to the satisfaction of the NRA and when GMP is in place. Each lot, if tested, should pass a test for unexpected toxicity.

A.5.8 Immunological activity
An immunological activity test (MIT, MICA, or approved alternative) should be carried out as described in section A.3.4.2.7, on each final lot, if such a test has not been conducted on the final bulk.

A.5.9 Inspection of final containers
Each container in each final lot should be inspected visually or mechanically, and those showing abnormalities should be discarded.

A.6 Records
The recommendations given in Good manufacturing practices for biological products (22) should apply.

A model of a suitable summary protocol to be used for pertussis vaccines is given in Appendix 6.
A.7  **Samples**

The recommendations given in Good manufacturing practices for biological products (22) should apply.

A.8  **Labelling**

The recommendations given in Good manufacturing practices for biological products (22) should apply, with the addition of the following:

- the words acellular pertussis vaccine;
- the word “adsorbed”;
- the name and address of the manufacturer;
- the recommended storage temperature and the expiry date if kept at that temperature;
- 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 should contain the following:

- a statement that the vaccine satisfies the recommendations 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;
- instructions for the use of the vaccine, and information on contraindications and the reactions that may follow vaccination.

A.9  **Distribution and shipping**

The recommendations given in Good manufacturing practices for biological products (22) 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. The recommendations given in the WHO Guidelines for stability evaluation of vaccines should apply (36).

Adequate stability studies form an essential part of vaccine development. The stability of the vaccine in its final containers, maintained at the recommended storage temperature, should be demonstrated to the satisfaction of the NRA.

For each of the antigens claimed to contribute to protective efficacy, real-time stability studies should support immunological activity and lack of specific toxicity of the product up to the expiry date.

The product must be manufactured in such a way that reversion to toxicity of the inactivated PT in the vaccine does not occur during the period of validity provided that the product is stored under the conditions stated on the label.

The desorption of antigens from the adjuvant, which may occur over time, should be investigated and, where possible, limits should be agreed with the NRA.

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

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

A.10.1.1 Stability for licensure

Studies that support stability of a vaccine for the purpose of licensure 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 tests for immunological activity. Tests should be conducted at appropriate time intervals during storage to determine the loss of immunological activity. Final containers from at least three lots of vaccine derived from different bulks should be tested on the expiry date to demonstrate stability during storage.

Accelerated stability data for products stored for limited periods at temperatures that may affect stability may support preliminary data from ongoing real-time stability studies but should not replace them. Any modification of the shelf-life approved as part of licensure requires additional stability data to support proposed modification and should be approved by the NRA. Following licensure, stability should be monitored throughout the proposed shelf-life.

A.10.1.2 Stability at different stages of the manufacturing process

Stability testing should be performed at different stages of production, namely single harvests, bulk materials, final bulk and final lot on at least three lots each. Suitable parameters indicating stability should be selected according to the stage of production. Manufacturers are encouraged to assign a shelf-life to all materials
during vaccine production, particularly intermediates such as single harvests, purified antigen bulk and final bulk.

A.10.1.3 Stability 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, for such vaccines under development, the stability data are generally available for a limited period. Appropriate documentation to support the stability profile of a vaccine should be submitted to the competent NRA 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 A.10.1 above, and approved by the NRA. For acellular pertussis vaccines, a temperature of 2–8 °C is generally considered to be satisfactory. This should ensure that the minimum potency specified on the label of the container or package will be maintained after release and 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 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 shelf-life supported by the stability studies, as described in section A.10.1 above, and approved by the NRA.

Part B. Nonclinical evaluation of acellular pertussis vaccines

Nonclinical testing of vaccines is a prerequisite for initiation of clinical studies in humans. There is no laboratory test or series of tests that will unequivocally assure that a newly developed acellular pertussis vaccine will be adequately safe and effective. With this limitation, these Recommendations describe a sequential approach to the collection of supporting evidence, beginning with a comprehensive programme of nonclinical testing, followed by a progression of clinical evaluations. This part describes the recommended nonclinical testing. The extent to which nonclinical studies will be required depends on the clinical experience that has already been gained with the different vaccine components. Animal studies, which aim to provide evidence that the vaccines induce functional
immune responses (e.g. induction of PT neutralizing antibodies, protection against bacterial challenge), form an essential part of the development of the vaccines. For vaccines containing acellular pertussis components that have not previously been evaluated for efficacy in clinical trials, the results of nonclinical testing represent only a part of the aggregate of data that needs to be considered when assessing the likelihood that the vaccine will prove to be effective when used in the clinical setting. Other considerations include manufacturing methods, control of the manufacturing process and clinical immunogenicity of the vaccine.

New vaccine formulations that have not been evaluated in safety and efficacy trials require extensive characterization, including assessment in vaccination/challenge studies in animal models (proof of concept) and safety testing in animals. However, extensive nonclinical testing may not be required for vaccines that use pertussis antigens that are the same (i.e. from the same manufacturer and produced by the same methods) as those in vaccines that have already been approved on the basis of their safety and efficacy.

For vaccines based on novel pertussis antigens or on formulations for which the pertussis components are produced using a new manufacturing process that is different from the established one, the characterization should include detailed evaluation and testing of: 1) the purified antigens prior to chemical treatment (e.g. detoxification), 2) the individual antigens prior to formulation, and 3) the formulated product. Although characterization is more difficult for pertussis antigens that are co-purified than for those that are individually purified, co-purified antigens should undergo similar evaluation and testing before and after chemical treatment (e.g. detoxification) and in the final formulated product.

Lots of individual antigens and formulated vaccine used in nonclinical studies should be adequately representative of those intended for clinical investigation and, ideally, should be the same lots as the ones to be used in clinical studies. If this is not feasible, then the lots used clinically should be comparable to those used in the nonclinical studies with respect to manufacturing, immunological activity, stability and other characteristics of quality. Details of the design, conduct, analysis and evaluation of nonclinical studies are available in the WHO Guidelines on nonclinical evaluation of vaccines (37).

B.1 Nonclinical characterization and testing of pertussis antigens and in-process materials

In the case of vaccines for which the acellular pertussis antigen is new (in terms of range of antigens and/or manufacturing processes for one or more antigens) an extensive preclinical evaluation should be undertaken. This should include thorough characterization of purified antigens before and after any chemical treatment, as well as any relevant in-process intermediates and final product materials. Characterization should evaluate purity, integrity and functional activity using a variety of approaches including physicochemical evaluation,
bioassays, measurement of residual toxicity/lack of reversion to toxicity, and active protection models. Given the complexity of acellular pertussis vaccines produced by co-purification methods, additional testing to fully define and specify the composition should also be conducted. For example, an assessment of the proportion of each antigen (e.g. the PT:FHA ratio) should be established to characterize clinical lots and to monitor product consistency.

The inclusion of a novel antigen – i.e. an antigen other than those that have already been tested in previous clinical efficacy trials (i.e. PT, FHA, PRN, FIM) – would require additional considerations. So would, for instance, the inclusion of the PRN antigen purified from B. bronchiseptica since this is not the same antigen as PRN from B. pertussis. When possible, the individual antigens should be evaluated in active protection animal models (16, 18, 19, 38, 39). Pertussis toxoid (PTxd) is effective in most active protection models, and therefore the demonstration of an additional benefit for antigens mixed with PTxd may be challenging. In such cases, the antigen should be examined in protection models with either no PTxd or suboptimal amounts of PTxd. The other antigens (FHA, PRN and FIM) are not necessarily active in all protection models, so it is important to consult the literature (see Table A4.1 below) to identify relevant models for each antigen.

The following describes the testing strategy that could be considered for the antigens that are contained in currently approved acellular pertussis vaccines, either produced by co-purification processes or by individual purifications of each component (PTxd, FHA, PRN, FIM2 and FIM3). When included in an acellular pertussis vaccine, the FIM2 and FIM3 are typically co-purified and processed as a single antigen (FIM2/3).

PT: purity and bioactivity of PT before chemical treatment, residual bioactivity after chemical treatment, lack of reversion to toxicity, activity in animal protection models, binding and functional activity of antibodies induced in animals, detection of known epitopes using monoclonal antibodies.

FHA: purity and functional integrity (e.g. haemagglutinating activity) of FHA before chemical treatment, residual activity after chemical treatment, activity in animal protection models, binding activity of antibodies induced in animals, detection of known epitopes using monoclonal antibodies.

PRN: purity of PRN, activity in animal protection models, binding activity of antibodies from immunized animals, detection of known epitopes using monoclonal antibodies.

FIM2, FIM3, or FIM2/3: purity of FIM, relative content of FIM2:FIM3, activity in animal protection models, binding and whole-cell agglutinating activity of antibodies from immunized animals and detection of known epitopes using monoclonal antibodies.
Special consideration should be given to vaccines based on genetically inactivated PT. Characterization studies of these vaccines should include evaluation of genetic stability of the production strain, consistency of the genetic sequence and attenuation of the toxic bioactivity (40).

Table A4.1 provides an overview of results from the published literature. The specific references should be consulted for details and additional information.

### Table A4.1

**Summary of published studies evaluating the ability of purified pertussis antigens to protect in mouse challenge models**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Respiratory challenge models</th>
<th>Intracerebral challenge models</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Purified antigen</td>
<td>Purified antigen</td>
</tr>
<tr>
<td></td>
<td>Active immunization</td>
<td>Passive immunization</td>
</tr>
<tr>
<td>PTxd</td>
<td>Yes References: 18, 19, 41–47</td>
<td>Yes References: 17, 43–51</td>
</tr>
<tr>
<td>FHA</td>
<td>Yes References: 18, 19, 44–46, 52–57</td>
<td>Yes References: 17, 44, 45, 51, 55</td>
</tr>
<tr>
<td>PRN</td>
<td>Yes References: 53, 58–61</td>
<td>? ?</td>
</tr>
<tr>
<td>FIM</td>
<td>Yes References: 45, 62–65</td>
<td>Yes References: 31, 65</td>
</tr>
</tbody>
</table>

PT = pertussis toxin; PTxd = pertussis toxoid; FHA = filamentous haemagglutinin; PRN = pertactin; FIM = fimbriae; “Yes” = protection was demonstrated; “No” = protection was not observed; “?” = no information was found.
Despite advances in knowledge regarding the mechanisms of toxicity of PT and other potentially reactogenic components produced by *B. pertussis*, uncertainty remains concerning the exact role played by these substances in the pathogenesis of pertussis and in vaccine reactions. This lack of information has hampered the establishment of scientifically sound limits for the residual activity of these components in vaccines containing pertussis antigens. However, vaccines containing chemically or genetically inactivated PT require thorough characterization to assess residual PT activity and, where appropriate, the possible reversion of this toxoid during storage. Manufacturers should demonstrate to the satisfaction of the NRA that chemically inactivated PT present in the final bulk does not revert to its toxic form before the vaccine expiry date. In addition, as part of the validation of the manufacturing process, manufacturers are required to submit evidence that the purification steps reduce the levels of LOS endotoxin, as well as the residual activities of heat-labile (dermonecrotic) toxin, tracheal cytotoxin and adenylate cyclase toxin, to acceptable levels.

In some countries, during development of the vaccine, the production process should be validated to demonstrate that it yields consistently an antigenic fraction that complies with the purity requirements listed below. After demonstration of consistency, the tests need not be applied routinely to each lot (66).

Adenylate cyclase: not more than 500 ng in the equivalent of 1 dose of the final vaccine, determined by immunoblot analysis or another suitable method.

Tracheal cytotoxin: not more than 2 pmol in the equivalent of 1 dose of the final vaccine, determined by a suitable method such as a biological assay or liquid chromatography.

Absence of residual dermonecrotic toxin: inject intradermally into each of three unweaned mice, in a volume of 0.1 ml, the amount of antigenic fraction equivalent to 1 dose of the final vaccine. Observe for 48 hours. No dermonecrotic reaction is demonstrable.

The mouse weight-gain test and the leukocytosis promotion test, which are currently used to monitor the toxicity of whole-cell pertussis vaccines, are considered to be of insufficient sensitivity to demonstrate residual PT activity in acellular pertussis vaccines. Specific tests for residual PT activity (see sections A.3.3 and A.3.4.2.5) are preferred.

### B.2 Nonclinical characterization and testing of final vaccine formulation

Given that there are currently no stand-alone acellular pertussis vaccines, the following studies should be carried out using the final formulation (i.e.
formulation that includes diphtheria and tetanus toxoid and other components). The capacity of an acellular pertussis vaccine to protect mice against a *B. pertussis* challenge may be used to establish a nonclinical proof of concept for new vaccine formulations. Two models have been developed to assess acellular pertussis vaccines: the MICA (Part A and Appendix 1) and the INCA (by instillation or by aerosol) (Part A and Appendix 5). However, as noted above (section B.1), PTxd is an effective antigen in both models; therefore, for vaccines that contain PTxd, the contribution of antigens other than PTxd may be difficult to discern in these models. Moreover, because residual active PT can influence the outcome of the MICA (32), care should be taken in interpreting the results of that assay. The assessment of functional antibodies, such as PT-neutralizing antibodies as evaluated in the CHO cell assay (for anti-PT) or whole-cell agglutinating antibodies (for anti-FIM), would provide further nonclinical evidence of the potential protective efficacy against *B. pertussis* in humans.

Additional toxicity and other testing should follow the recommendations outlined in the WHO guidelines on nonclinical evaluation of vaccines (37).

**Part C. Clinical evaluation of acellular pertussis vaccines**

This part of the Recommendations provides guidance on issues related to the design and evaluation of clinical studies. Most studies are expected to be comparative in nature, so the choice of a comparator vaccine is discussed in some detail. Importantly, only those vaccines with adequate nonclinical testing should be considered for clinical evaluation, with the local NRA being responsible for evaluating adequacy of nonclinical information. Because efficacy trials appear very difficult, the trials for assessing safety and immunogenicity are emphasized.

Clinical evaluations conducted over the past 30 years provide models for the clinical evaluations of new vaccines. Most importantly, in the period between 1986 and 1996, several acellular pertussis-containing vaccines, including both vaccine types (vaccines composed of purified antigens and vaccines composed of co-purified antigens) were evaluated in a series of efficacy trials. In the first acellular pertussis vaccine efficacy trial in Sweden (1986–1987), a PTxd and a PTxd/FHA vaccine from the same Japanese manufacturer were evaluated (67). The efficacy estimates for the primary case definition (culture-confirmed pertussis with at least one day of cough) were 69% (95% CI 47–82) for the two-component vaccine and 54% (95% CI 26–72) for the PTxd alone. Secondary analyses of this trial revealed the critical importance of case definition, in particular the marked influence on vaccine efficacy estimates of the laboratory and clinical criteria used to define a case. For example, markedly different efficacy estimates for the one- and two-component vaccines could be obtained depending on whether or not mild
clinical cases were included (68). To address the problems with case definition, WHO convened an expert group in 1991 to recommend case definitions that could be used for subsequent efficacy studies (69). The recommended primary case definition required 21 days of paroxysmal cough and laboratory confirmation by culture, serology or household contact with a confirmed case. However, because this primary case definition provided incomplete information, evaluation of secondary end-points was strongly encouraged. The evaluation of efficacy against milder illness (e.g. fewer than 21 days of paroxysmal cough) was considered of particular importance.

Additional trials were conducted between 1991 and 1996 (11, 12, 70–79). In these trials, DTaP vaccines containing 1–5 pertussis components were investigated. Different study designs were employed in these trials, namely: 1) randomized placebo-controlled cohort trials, 2) household contact studies, and 3) case–control studies. The different calculated vaccine efficacies were affected by study design as well as case definition. The most reliable estimates of absolute vaccine efficacies were obtained for those trials that used a double-blind format with an unvaccinated control group (80). Blinding was not possible in the case–control studies and in most of the household contact studies, and thus the efficacy estimates for such trials have more potential for bias. The exceptions were household contact studies which were nested within some randomized controlled cohort trials.

This series of trials revealed that all the tested acellular pertussis vaccines protected children against pertussis to at least some degree (11, 12). However, unless the vaccines were tested in parallel within the same trial, comparing the efficacy of the different acellular vaccines must be done with caution, as all the trials varied with respect to design, case ascertainment methodology, and case definition. For instance, in placebo-controlled cohort trials, culture-confirmation was more likely to occur in unvaccinated than in vaccinated subjects, leading to inflated vaccine efficacy estimates. This bias was overcome to a great extent by employing appropriate serological tests to confirm the cases. Similarly, mild cases were proportionally more frequent in vaccinees than in controls; thus efficacy estimates were inflated when milder cases were excluded or were deflated when they were included. Some of the randomized placebo-controlled cohort trials investigated two different acellular pertussis vaccines and, from these, some comparisons could be made. In two studies, an acellular pertussis vaccine containing five components provided better protection than the specific (and never licensed) two-component vaccine included in that trial (72, 75). However, the vaccine composition that optimally protects against both mild and severe disease remains uncertain. Epidemiological investigations have shown that disease has been controlled by vaccines of varying composition (1, 10, 81, 82).
Several of these trials included both whole-cell and acellular pertussis vaccines. Some tested whole-cell vaccines provided less protection than the acellular vaccines (71, 72). However, in other trials other whole-cell vaccines appeared to be more efficacious than most acellular vaccines, particularly against mild pertussis (75, 77). Heterogeneity among whole-cell vaccines has been reported since the 1950s, and emphasizes the importance of monitoring the efficacy of any whole-cell or acellular pertussis immunization programme.

Two of the efficacy trials were designed to determine the antibody values at the time of exposure, and thus were able to evaluate whether the presence of specific antibodies was correlated with protection from disease (13, 14). Both studies showed that the presence of antibodies to PRN, PT and fimbriae correlated with protection. Neither study, however, found a correlation with antibody to FHA. However, a role for an immune mechanism other than serum antibody cannot be ruled out for this antigen.

Following the completion of these trials, many countries began exclusive use of acellular pertussis vaccines. Several studies have attempted to evaluate the duration of protection (82–84). To date, studies support a conclusion that efficacy is retained for at least five years after a three- or four-dose series of acellular pertussis vaccine. Further evaluations should be able to define the duration of protection more clearly and thus provide guidance to public health officials on the optimal time for administration of booster doses.

With respect to safety, several head-to-head studies have demonstrated that primary immunization with DTaP vaccines caused fewer local reactions and less fever than DTwP vaccines (11, 12). However, no clinically significant differences in safety have been demonstrated among acellular pertussis vaccines with differing numbers of components. Several studies evaluating booster doses have indicated that the frequency of significant redness and swelling (e.g. redness and/or swelling greater than five centimetres or swelling of the entire limb) increases in those subjects who have received multiple doses of DTaP vaccines. With respect to more serious events, the literature provides no reliable basis for a causal relationship between vaccination and the handful of other serious adverse effects described in case reports or national adverse event reports (12).

C.1 General considerations for clinical studies

This section addresses some issues that are specific to, or particularly relevant to, the clinical development of new acellular pertussis vaccines. The recommendations made should be considered in conjunction with the general principles described in the WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (85).
These Recommendations should be viewed in the light of further data on the safety, immunogenicity and effectiveness or any relevant data on other types of acellular pertussis vaccines that may become available in the future.

Manufacturers should provide justification for the choice of the vaccine formulation (e.g. the pertussis components included in the formulation) and the design of the clinical development programme used to evaluate the vaccine, including the size of studies and the end-points for evaluation.

For vaccines that contain new acellular pertussis vaccine components rather than established components – with established components defined as the same pertussis purified antigen(s) manufactured by the same company, using the same process, and formulated in the same way as components tested in clinical efficacy studies – the immunogenicity data obtained in clinical studies should be considered along with manufacturing information and nonclinical data when assessing the likelihood that the vaccine will prove to be effective in the clinical setting. Clinical investigations should be initiated only with vaccines that have undergone thorough nonclinical evaluations, as described in Part B. Consistency of manufacturing for the vaccine lots used in clinical trials should be demonstrated and well documented. It is expected that several lots with the same formulation intended for marketing will be used in the late stages of the clinical development programme.

C.1.1 Scope of the studies

Placebo-controlled protective efficacy studies are no longer feasible for ethical reasons, and trials designed to measure efficacy relative to that of a licensed acellular pertussis vaccine with proven efficacy would need to be very large in order to provide adequate precision in the relative efficacy estimates. Therefore, the approval of any new acellular pertussis vaccines would be likely to rely on data from comparative immunogenicity and safety trials of adequate size and design to provide reasonable assurance of their clinical benefit. When applicable and when relevant, studies documenting the performance of the investigational vaccine when co-administered with other routinely recommended infant and toddler vaccines should be performed.

Infants less than three months of age are at highest risk of hospitalization and death from pertussis. There is a growing interest in approaches that could provide improved protection for these very young infants. Such approaches, include, for example, the development of stand-alone acellular pertussis vaccines to be used as a birth dose and the immunization of mothers before or during pregnancy. However, the unique considerations that apply to the clinical evaluation of vaccines in these special populations are not included in this document.
C.1.2 Comparator vaccine

The predictive relationship between the concentration of antibody induced and protection against pertussis has not been established for each antigen. Therefore, one of the critical aspects when designing clinical trials for licensure is the choice of a comparator vaccine. When doing this, the points made below should be considered.

The choice of the comparator vaccine will vary according to the characteristics of the new acellular pertussis vaccine under development. However, the comparator should in general be the vaccine most similar to the new vaccine with respect to content and composition of the acellular pertussis component. Three potential scenarios are proposed when assessing a new vaccine formulation:

- **Scenario 1** – the new acellular pertussis combination vaccine contains an established acellular pertussis component (i.e. same purified pertussis antigen(s) manufactured by the same company using the same processes, and formulated the same way) that has been found suitably efficacious in a clinical efficacy trial. In this scenario, the most appropriate comparator vaccine would be the most similar licensed product from same manufacturer. This scenario applies to the evaluation of different combination vaccines based on the same DTaP components (e.g. DTaP-IPV, DTaP-HepB, DTaP-HepB-Hib etc.) or DTaP with different amounts of pertussis components (e.g. booster formulations Tdap). The evaluation of a combination vaccine in this scenario is based on a non-inferiority clinical trial of immune responses relative to the separately administered licensed DTaP or DTaP-based combination vaccine.

- **Scenario 2** – the new acellular pertussis-containing vaccine has a composition which is the same as, or very similar to, that of an established acellular pertussis component that has been found suitable in a clinical efficacy trial. However, some or all antigens are made by a different manufacturer and/or by a different process than the vaccine tested in a previous protective efficacy study. In this scenario, the most appropriate comparator would be the licensed product (with proven efficacy) with matching composition (same acellular pertussis antigens, similar amounts).

**NOTE:** Vaccines evaluated in efficacy studies include one-component (PTxd) (67, 70), two-component (PTxd/FHA) (67, 72, 74, 75, 77), three-component (PTxd/FHA/PRN) (71, 73, 75), four-component (PTxd/FHA/PRN/FIM2) (78), and five-component (PTxd/FHA/PRN/FIM2/FIM3) (72, 75) acellular pertussis vaccines.
Scenario 3 – the new acellular pertussis-containing vaccine has an acellular pertussis antigen composition that is not the same as that of an already licensed acellular pertussis vaccine that has been found suitably efficacious in a clinical efficacy trial. There are at least two ways this could occur, namely: 1) the vaccine could be based on the currently used antigens but present in different combinations such as PTxd/PRN or PTxd/PRN/FIM2/FIM3, or 2) the vaccine could include novel antigens in combination with one or more of the currently used antigens. In this scenario, the most appropriate comparator would be the licensed product (with proven efficacy) with the most similar composition.

It is important to highlight that, for scenarios 2 and 3, manufacturers should justify the choice of comparator vaccine and the non-inferiority margin used, particularly when there are differences in the content and composition of the acellular pertussis components.

C.2 Assessment of immune responses

C.2.1 Assays to assess antibody responses

Serological assays used in clinical immunogenicity studies in support of vaccine licensure require validation (85). An international reference pertussis antiserum has been established to assist in the standardization of serological methods (86). Thus, to ensure the comparability and acceptability of the serological data across trials, results of immunogenicity should be expressed in IU in reference to this International Standard for pertussis antiserum. A rigorous assessment of assay specificity is essential prior to initiating validation studies. Formal validation should assess all appropriate performance criteria – including accuracy, linearity, precision and range – and robustness studies are also recommended. The validation studies should be designed to demonstrate that the range (including the lower limit of quantitation) is suitable for the clinical study, and should consider the way in which the vaccines are to be compared to each other (e.g. whether the criteria for evaluation are based on percentages with post-primary series titres above a threshold, seroconversion rates or geometric mean antibody titres).

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. Suitable assays are unlikely to be commercially available.

Specific antibody responses to different components of the vaccine (e.g. PT, PRN, FIM etc.) can be assessed by methods that measure the concentration
of antibody binding to a specific antigen (e.g. ELISA) or, when applicable, the functional biological activity by measuring the PT neutralizing titre (e.g. CHO cell assay) (87) or the *B. pertussis* agglutination titre (88–90).

Cell-mediated immune (CMI) responses play a role in protecting against *B. pertussis* infection. However, immunological assays to evaluate CMI responses following immunization have not been standardized and have not been used to support licensure. Nevertheless, the exploratory assessment of CMI should be encouraged in order to enlarge the body of knowledge regarding all aspects of the immune response to pertussis antigens.

### C.2.1.1 ELISA to assess antibody responses to acellular pertussis components

The assessment of antibody responses to specific pertussis components included in the vaccine should be regarded as the primary means of assessing the immune response to new acellular pertussis vaccines. The standardization of serological methods for *B. pertussis* has been pursued not only for the purpose of licensure of new pertussis vaccines but also for clinical diagnosis of pertussis infection and for seroepidemiological studies (91). However, it is important to note that assays developed and optimized for diagnostic and epidemiological purposes, including most commercially available ELISA kits, are unlikely to have the performance characteristics needed for vaccine immunogenicity studies. For instance, diagnostic kits are unlikely to provide the specificity required to assess each of the pertussis components individually (e.g. PT, FHA, etc.) and the accuracy to determine geometric mean concentration (GMC) (92).

Collection, recording, analysis and interpretation of data should be conducted according to good clinical practice guidelines (93). Methodological and statistical considerations described in WHO guidelines should be taken into account (85).

### C.2.1.2 Assessment of functional antibody titres

Functional activities of antibodies against pertussis components have been identified as important additional parameters to consider, particularly when evaluating new formulations containing PTxd and FIM which are known to induce antibodies with functional activity such as toxin neutralization and bacterial agglutination respectively (89). Assays to measure PT neutralizing and whole-cell *B. pertussis* agglutinating antibodies have been established (87, 88, 90). Although no functional thresholds have been found to correlate directly with the protective efficacy of pertussis vaccines, there are nevertheless important immune parameters to determine as part of the overall comparison of new vaccine formulations to those proven to be safe and effective.

When feasible, functional antibodies should be measured, at a minimum, in a subpopulation of the comparator and test vaccine groups.
C.2.2 Criteria for evaluation of immune responses

The preferred method for evaluating new vaccine formulations is the direct clinical comparison of licensed vaccines with proven pre-licensure clinical efficacy, with the new product through randomized controlled trials.

C.2.2.1 Primary immunization of infants and young children

In comparative studies of post-primary immune responses, the main analysis will be based on demonstrating that the response in subjects immunized with the test vaccine is not inferior to that in the comparative vaccine group(s). The selection of the primary parameter for the assessment of non-inferiority, the predefined margin of non-inferiority and hence the total sample size for a comparative study, will need careful justification (85). Although studies that compare immune responses between candidate and licensed acellular pertussis vaccines are essential, comparisons with historical data that were generated during previous protective efficacy studies using similar assays may be used to provide supportive evidence. While a demonstration that the new candidate vaccine is immunogenic in humans is important, the data should be interpreted with some caution. In particular, when evaluating immunogenicity data comparing vaccines produced by different manufacturers or produced using different methods, equivalent efficacy cannot be directly inferred from equivalent immunogenicity.

The study objectives must be taken into account when defining appropriate time intervals for assessing the immune response. In most cases, clinical studies for new vaccines for infants are designed to determine the antibody response to acellular pertussis components at approximately four weeks following the final dose. Predefined non-inferiority criteria using an appropriate acceptability limit are used to compare the responses in subjects immunized with the test vaccine versus the licensed comparator vaccine (see section C.1.2) using the end-points described below.

The following co-primary analyses are recommended:

- **Percentage of responders** – in one primary analysis, the percentage of responders with a significant increase (e.g. fourfold increase) above pre-immunization for each of the acellular pertussis components is compared between subjects immunized with test vaccine and the licensed comparator vaccine. Alternative definitions for responders could be considered if well justified. The groups should be compared using an appropriate predefined non-inferiority limit; generally the lower bound of the two-sided 95% confidence interval of the observed difference should not be less than the criterion approved by the NRA, most commonly 10 percentage points.
- **Magnitude of the response** – in a second primary analysis, the magnitude of the response is compared – on the basis of the GMC induced by the new vaccine and the licensed comparator using a predefined margin of non-inferiority – to evaluate the antibody response (GMC ratios) to each acellular pertussis component. The lower bound of the two-sided 95% confidence interval of the observed ratio of the GMC of the new vaccine relative to the control should not be less than the criterion approved by the NRA (most commonly 0.50 or 0.67).

In case of failure to meet the predefined non-inferiority criteria, detailed investigation of the immune response and the reasons for not meeting the criteria may be considered. In particular, the NRA may take into consideration the results from the antibody responses to each of the antigens, as well as any differences in composition between the test and the comparator vaccines, and the available information about the contribution of that antigen (i.e., the antigen to which the antibodies are directed) in protection.

C.2.2.1.1 **Secondary analysis**

**Functional antibody response.** When evaluating new vaccine formulations, it is important to assess as many immune parameters as possible. Therefore, functional antibody responses (i.e. PT neutralizing titres or *B. pertussis* agglutination titre if PTXd or FIM are part of the formulation, respectively) should be determined in a randomized subset of vaccinated subjects within some or all of the clinical studies. At present, the interpretation of functional antibody data is made difficult by the fact that a titre that might correlate with protection against pertussis infection is unknown. For this reason it is recommended that comparisons of functional antibody titres between the new vaccine and the licensed comparator should focus on GMT ratios.

C.2.2.1.2 **Additional information**

**Reverse cumulative distribution (RCD) curves (94).** Use of RCD curves which display the accumulated proportion of individuals with an antibody concentration greater than or equal to a given level have been shown to be useful when comparing the response to the test and licensed comparator vaccines. RCD plots should be generated for ELISA data and for functional antibody response. The review of these data should be viewed as exploratory.

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

C.2.2.2 Booster immunization of older children, adolescents and adults

In most instances, the emphasis for initial use and evaluation will be for the primary immunization of infants. However, acellular pertussis vaccines may also be used for booster doses in the second year of life, and in preschool children, adolescents and adults. Currently, different immunization schedules are employed in different countries for primary and booster immunization. In all cases, the chosen schedule should be supported by appropriate immunogenicity studies. Previous experience has demonstrated that vaccines intended solely for booster immunization may require lower amounts of one or more of the pertussis antigens.

An active comparator vaccine may not be needed when evaluating the immunogenicity of the acellular pertussis components used as a booster vaccine, such as in older children and adults. In such cases, it may be possible to compare the immune response of adolescents and adults following a single dose to that of infants who received primary immunization with the corresponding DTaP vaccine (historical comparator). In addition, the ability of these vaccines to induce immunological memory assessed by an anamnestic response following immunization should be evaluated for each acellular component.

C.2.3 Combined vaccines and concomitant administration with other vaccines

C.2.3.1 Combined vaccines

In the case of combination of acellular pertussis components with other antigens, potential interference between the pertussis components and the other antigens and/or excipients should be investigated, as described in the WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (85).

In particular, it has been demonstrated that some acellular pertussis components can have a negative impact on immune responses to some polysaccharide conjugated antigens when administered in pre-formulated products or when vaccines are mixed only immediately before injection (e.g. Haemophilus influenzae type b conjugate vaccine responses in some combination products containing PRP-T and some acellular pertussis components). Therefore, the immune responses to all the antigens in the final combined formulation should be shown to be satisfactory through well-designed randomized comparative trials. If there is any immune interference observed with respect to any of the combined antigens, the possible clinical implications should be carefully considered before proceeding with clinical development.
C.2.3.2 Concomitant administration with other vaccines

In recent years, it has become apparent that concomitant administration of acellular pertussis components with other vaccines in routine use, including conjugated vaccines, may give rise in some situations to detectable immune interference, although the clinical significance of the observed phenomena is not always clear. Examples include decreased antibody responses to *Haemophilus influenzae* type b conjugate vaccine and to meningococcal C monovalent vaccine. Thus it is important that immune responses to candidate acellular pertussis vaccines should be evaluated on co-administration with other vaccines that are representative of types that, for convenience and compliance reasons, are very likely to be given at the same clinic visits. Responses to other co-administered antigens should also be evaluated. The approach to these studies is based primarily on demonstrating non-inferiority of responses to antigens when vaccines are co-administered, compared to each vaccine given alone, with careful justification of predefined non-inferiority margins.

These studies might compare concomitant administration with administrations made in a staggered fashion (e.g. together at two, four and six months compared to the usual antigens at this schedule and the new vaccine at three, five and seven months).

C.3 Safety evaluation

As stated in section C.1.1, placebo-controlled efficacy studies which would also deliver a large safety database are not feasible. Nevertheless, the pre-licensure assessment of vaccine safety is a critically important part of the clinical programme, and should be developed to meet the general principles described in the WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (85). A comparison of rates of adverse events between test and approved comparator vaccines is commonly a predefined secondary end-point in study protocols. The minimum acceptable size of the safety database at the time of approval should take into account the vaccine composition (including all antigens and adjuvants), the presence of novel antigens, and any past experience with vaccines with the same or similar composition of the acellular pertussis component.

For new vaccines, a total safety database (combined from all trials in the same targeted age group) of approximately 3000–5000 subjects is commonly expected because this allows for the evaluation of uncommon adverse events – i.e. those that occur at a rate between 1 in 100 and 1 in 1000 subjects (85). However, depending on the data available for the vaccine, the NRA may accept a smaller number or may request a larger database prior to first approval.

Information on adverse events such as extensive limb swelling syndrome should be carefully monitored in studies evaluating the safety of booster doses.
C.4 Post-marketing studies and surveillance

Every effort should be made to improve current scientific understanding of the protection in humans afforded by acellular pertussis vaccines by providing data from active post-marketing surveillance. Vaccine effectiveness in the population should be reported wherever possible. In addition, given that limited safety data are obtained in pre-licensure studies, all relevant safety-indicating parameters should be monitored as part of post-marketing surveillance programmes. In particular, the impact of routine vaccination on pertussis infection needs to be assessed in comprehensive studies of vaccine performance. Ongoing surveillance programmes should be in place to monitor for longer-term protection and for evidence of any changes in vaccine effectiveness.

In reality, sound and comprehensive safety and effectiveness data cannot be collected by the manufacturers alone. Therefore, there should be discussions between vaccine manufacturers responsible for placing the product on the market and national and international public health bodies regarding the feasibility of estimating effectiveness and safety in the post-marketing period. Reliable estimates of effectiveness can be obtained only in geographical locations where appropriate vaccine campaigns are initiated and where there is already a suitable infrastructure in place to identify cases of pertussis disease.

General WHO guidelines for continued oversight of vaccines after licensure should be followed (85). All data collected should be submitted to the responsible NRAs at regular intervals so that any implications for the marketing authorization can be assessed.

Part D. Guidelines for NRAs

D.1 General

The general recommendations for control laboratories contained in the Guidelines for national authorities on quality assurance for biological products (95) and Guidelines for independent lot release of vaccines by regulatory authorities (96) should apply.

The detailed production and control procedures and any significant changes in them should be discussed with and approved by the NRA.

Consistency of production has been recognized as an essential component in the quality assurance of acellular pertussis vaccines. In particular, the NRA should carefully monitor production records and quality control test results for clinical lots as well as a series of consecutive lots of the final bulk.

D.2 Official release and certification by the NRA

A vaccine 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 NRA 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 Recommendations. The certificate should state the number under which the lot was released by the NRA, 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 Appendix 7.

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Appendix 1

Modified intracerebral challenge assay

1. Materials

Strain 18323 of *Bordetella pertussis* (hereafter referred to as the challenge strain in this appendix) should be used for challenge. The diluent for the test sample and standard should be sterile physiological saline (0.85% NaCl).

The challenge strain should be cultured on Bordet–Gengou medium for approximately 24 hours and suspended in 1% w/v casamino acid solution containing 0.6% w/v of sodium chloride (pH 7.0–7.2) to a concentration of approximately 200 LD$_{50}$ per 0.025 ml (or approximately 1×10$^5$ organism/challenge dose) to serve as the suspension for challenge. Alternatively, a stable frozen stock can be used for direct challenge after appropriate dilution.

2. Test procedures

The test sample and standard should be diluted serially to make at least three levels of fourfold or other suitable logarithmic dilutions. Each dilution should be given by intraperitoneal injection at a dose of 0.5 ml to at least 16 mice aged approximately four weeks. The animals should be of the same sex or both sexes in equal numbers for each dose. The challenge suspension should be given by intracerebral injection into the animals at a dose of 0.025 ml 21 days after immunization. The animals should be observed for 14 days. Any animals dying within three days after challenge should be excluded from the test. Any animals showing paralysis or swelling of the head at the end of the observation period should be counted as deaths.

At least three appropriate serial dilutions of the challenge suspension should be injected into a group of at least 10 mice to titrate the virulence. The bacterial count for the LD$_{50}$ per 0.025 ml of the challenge suspension should be no more than 300 CFU.

3. Criterion for judgement

Assay data are analysed using parallel line analysis following probit transformation of the proportion of mice responding. The dose–response curves of the test and reference vaccines are checked for significant deviations from linearity and parallelism. If there is a significant ($p < 0.05$) regression of probit response on log dose and there are no significant deviations ($p > 0.05$) from linearity or parallelism, the potency and its 95% limits are calculated. The First WHO International Standard for acellular pertussis vaccine (JNIH-3) has been
established and an in-house reference used in this test should be calibrated in terms of the International Standard. The specifications should be established with the agreement of the NRA. Where used currently, the potency of the test sample is considered to be passed if it is no less than 8 IU/ml (4 IU/human dose) upon statistical analysis.

4. Retest

If a test vaccine did not pass in the first test, the test should be repeated using the same number or an increased number of mice. Results for all statistically valid assays should be combined. Weighted mean log potency should be calculated for homogeneous results using log potencies obtained in repeated tests using inverse of variance estimate for each log potency value as weight.
Appendix 2

Histamine sensitization test by temperature measurement

Groups of mice (with no fewer than 10 mice each) defined with respect to strain, sex and age, should be randomly allocated to the different treatments. Samples should include the test sample(s) and a Reference Preparation. If reversion to toxicity testing is required by the NRA the test also should include a sample of the test vaccine incubated at 37 °C for four weeks. All mice should be challenged post-sensitization with histamine dihydrochloride. The reference and histamine dihydrochloride should be diluted with physiological saline. If PT is used as reference, physiological saline or phosphate buffered saline, both containing 0.2% (w/v) gelatin, should be used as diluent to prevent possible loss of PT activity by adsorption to the container. Appropriate thermometers with recommended precision of 0.1 °C and capable of measuring temperatures between 25 °C and 40 °C should be used for the test.

The Reference Preparation should be diluted to give a suitable dose-response. The test sample (usually a single human dose) and each dilution of the Reference Preparation in a volume of 0.5 ml should be given by intraperitoneal injection to each group of mice. Four days after injection, 4 mg of histamine dihydrochloride should be intraperitoneally injected into each mouse. The rectal or dermal temperature should be measured 30 minutes after histamine injection for all mice. Temperature should be recorded for all mice, including those that die within the 30-minute observation period.

Temperature responses are analysed using a suitable statistical method to give an estimate of the residual activity of PT in the test vaccine, in relation to the Reference Preparation.

The test lot passes the test if the estimated residual activity of PT in the test group is not higher than the value specified by agreement with the NRA. There is currently no internationally agreed upper limit for active PT in acellular pertussis vaccines. In some countries an upper limit of 1.09 or 2.18 IU (0.2 or 0.4 HSU) of PT per single vaccine dose is a requirement for DTaP vaccines.
Appendix 3

Histamine sensitization test by lethal end-point assay

Groups of mice, each of an appropriate number defined with respect to strain, sex and age, should be randomly allocated to the different treatments. For assays performed for validation purposes and initially after vaccine licensure, the positive control set consists of groups that should be injected intraperitoneally with three or more serial dilutions of a Reference Preparation of PT of suitable specific activity (IU/ng). The dilution factor should be chosen so as to obtain a graded response; however, it should be no greater than five. An additional group of mice (the negative control group) should be injected intraperitoneally with diluent. One group should be injected intraperitoneally with the test vaccine and, if reversion to toxicity testing is required, another group should receive the test sample incubated at 37 °C for four weeks. A single human dose (some methods allow up to two single human doses) of the final bulk is used as the test dose for both groups. The position of the cages on the shelves during the testing period should be allocated at random in order to reduce the influence of positional effects on the assay outcome. All mice should be challenged by injection with a defined dose of histamine (1 or 2 mg of histamine base is most commonly used) at four or five days after sensitization or injection with diluent. The histamine challenge dose may be administered intraperitoneally or intravenously; however, the injection route should be defined and the same route should be used for all testing within the laboratory. Histamine challenge should follow the place order of the cages on the shelves. Deaths within 24 hours of histamine injection should be recorded.

The assay sensitivity and other validity criteria should be defined in agreement with the NRA. For the assay to be considered valid, mice injected with diluent (negative control) must not show, in general, sensitization to the lethal effect of histamine. However, experience has shown that, with low frequency, a small percentage of mice (i.e. less than 5%) in the negative control group may die following histamine challenge. Thus some laboratories consider a test valid if there is no more than one death in a negative control group of 20 or more. Each test should also meet the criteria set to demonstrate its sensitivity. Several strains of mouse (all with Swiss-Webster ancestry) are highly responsive to histamine sensitization but a number of strains, both inbred and outbred, are weakly responsive. The susceptibility to sensitization of the strain chosen for the test should be defined during assay validation studies and approved by the NRA. Adequate susceptibility of mice used in a HIST should be verified by demonstrating that the sensitizing dose of the PT control meets criteria established during assay validation studies and approved by the NRA.
validation. Once linearity has been established by repeated experiments using multiple doses of a control PT, a suitable dose of the reference toxin, chosen in the linear region of the dose–response curve and giving a positive response considered appropriate by the NRA, should subsequently be included in each assay as the positive control group to demonstrate assay sensitivity.

In some laboratories, the test also includes a reference group of mice injected with PT at a dose previously set as the allowable upper limit of PT in the product or with a reference vaccine with established clinical safety.

A lot passes the test if the proportion of animals that die following sensitization with the test dose of vaccine and the subsequent histamine challenge do not surpass the maximum proportion approved by the NRA. This proportion should be related to the performance in the test of lots shown to be safe and effective in clinical trials or those used in support of licensure. When a reference group is included, the lot passes the test if the percentage of deaths in the test group is not greater than that in the reference group.

If a vaccine lot fails in a single test, it should pass two additional, consecutive and independent assays to be considered suitable for release.
Appendix 4

Mouse immunogenicity test

The mouse immunogenicity test (MIT) for an acellular pertussis vaccine is an assay designed to demonstrate consistency between vaccine lots on the basis of the induction of antibody in mice by each antigen in the vaccine. This test is product-specific and a suitable product-specific reference (or control) vaccine is required for a meaningful assay. Immunogenicity can be measured either in terms of the amount of antibody produced in mice injected with a defined test dose, or as the dose of antigen that induces a defined measurable antibody response in a certain proportion of mice (e.g. the median effective immunizing dose, ED50).

For each antibody, the linear-response region of the dose–response curve (vaccine dose versus antibody production) should be determined. In the first method, a group of mice is injected once with a preselected dose of vaccine that is within the linear-response region. For preparations containing multiple pertussis antigens, more than one test dose of vaccine, and therefore more than one group of mice per lot, may be required because of the differential immunogenicity of these antigens in mice. In the second method, groups of mice are injected with a suitable range of dilutions of vaccine, and the proportion of responding animals is determined at each dose. After consistency in manufacturing and testing has been demonstrated to the satisfaction of the NRA, the serial-dose method may be simplified to an appropriate single-dose (e.g. ED50 for the antigen) assay.

Regardless of test design, the antibody content of test sera is calculated relative to a stabilized reference serum by means of a validated ELISA.

For all antigens, reproducibility of the antibody response in the chosen strain of mice should be verified in every test by the inclusion of group(s) of mice injected with homologous reference (or control) vaccine. The reference (or control) vaccine ensures that the test mice respond in a way that is consistent with previous testing. The stability of the reference (or control) vaccine should be monitored. Appropriate stabilization of the reference (or control) vaccine, preferably by lyophilization, is recommended. An example of conditions for lyophilization that have been successfully used are as follows: 3.5% polygelin (1:1) under freeze-drying cycle at −50 °C load, −50 °C freeze over 2.5 hours, then primary drying at 35 °C (100 µbar vacuum) and secondary drying at 30 °C (30 µbar vacuum). The reference vaccine does not need to be a clinical lot because acceptance criteria are values reflecting the behaviour in the test of clinical lots or those lots used in support of licensure, either in absolute terms or in terms relative to the reference vaccine. However, the reference vaccine should be sufficiently similar to the clinical lots in composition and manufacture to
serve as an adequate control in the test. The response of the test vaccine may be reported either in absolute terms or in terms relative to the reference vaccine. Calibration of replacement reference vaccines for the MIT should make use of sound statistical principles to prevent drift in the efficacy of acellular pertussis vaccines in the market.

The specifications for evaluating vaccines containing acellular pertussis are product-specific and are based on an appropriate statistical analysis of the responses observed in the MIT test for clinical vaccine or other lots used in support of licensure. Specifications must be carefully justified and defined with the agreement of the NRA. Specifications should be defined for each antigen claimed to contribute to vaccine efficacy. Specifications based on a simple failure to reject the null hypothesis of equivalency of immunogenicity between a reference lot and a manufacturing lot, or between two consecutively manufactured lots, are not recommended.

Two components of the test require careful attention:

**Mouse:** strains of mouse (if necessary more than one) should be selected so that a sufficient antibody response is obtained for each antigen. The optimal age for mouse immunization (e.g. more than five weeks of age), the optimal time for bleeding (e.g. 4–6 weeks after immunization), and the isotype of the antibody response should be thoroughly studied. The test design should be agreed with the NRA.

**Antibody detection system:** the ELISA used for the detection of antibodies should be subjected to thorough validation and standardization studies. These studies should include determination of the biochemical integrity and immunological purity of antigens used for coating assay plates and determination of the optimal antigen-coating concentration. For this purpose, the production and standardization of a working-reference mouse serum is of utmost importance. Calibration of the working-reference mouse serum in terms of the international reference serum (97/642) may provide a suitable control and facilitate inter-laboratory comparisons. Studies for reference serum standardization should include an evaluation of the parallelism of the titration curves of reference and test sera. Another component of the antibody detection system requiring careful study is the anti-mouse-immunoglobulin-enzyme conjugate. This reagent should be characterized in terms of isotype specificity and subclass reactivity, and a suitable working dilution should be determined.

The reproducibility (intra-assay and inter-assay) of the assay for sera containing different amounts of antibody and the limits of detection and quantitation (LOD and LOQ, respectively) should be studied.

The development of criteria for acceptance of a vaccine lot subjected to the immunogenicity test should take into account the following ELISA validity criteria:
The average absorbance value for normal mouse serum should be below a historically defined upper limit. Normal mouse serum should be obtained from mice injected with diluent and housed with vaccinated mice for the duration of the immunization period. The absorbance of normal mouse serum should be measured in the same ELISA as the sera of immunized mice.

The parameters of the curve relating absorbance to dilution for the reference serum should be within historically defined upper and lower limits.

A control serum with characteristics similar to the test sera and stored in a separate location from the reference serum should be included in every ELISA plate. The ratio of the ELISA units calculated for the control serum to those for the reference serum should be within historically defined upper and lower limits.

If the ELISA meets these validity criteria, the antibody values should be calculated for mice immunized with the reference (or control) vaccine and the test vaccine. Sera with ELISA unit values below the LOQ should be qualified as belonging to non-responder mice. For the purpose of calculating geometric mean antibody level, an arbitrary value (e.g. 1/2 LOQ) may be assigned to such sera. Alternatively, the geometric mean antibody level could be calculated using only those values above the LOQ, provided a limit is in place for the minimal acceptable number of values to use for the calculation. If immunogenicity is being expressed in terms of dose of vaccine, then the number of mice responding to each antigen is used to calculate the ED\textsubscript{50}. If the ELISA validity criteria are not met, the ELISA should be repeated.

After either a geometric mean or ED\textsubscript{50} has been calculated for the reference (or control) vaccine, the value should be compared with the criteria for sufficient antibody response that were established when the assay was validated. If these validity criteria are met, the results for the test vaccine should be evaluated as described below. If the validity criteria for the reference vaccine are not met, the ELISA should be repeated on all sera (from mice inoculated with both reference and test vaccine). If the criteria are not met after a second ELISA, immunization should be repeated.

To pass the immunogenicity test, the geometric mean antibody levels or ED\textsubscript{50} for mice immunized with test vaccine should meet the criteria that were established when the assay was validated. Alternatively, immunogenicity of the test vaccine can be expressed relative to the immunogenicity of the reference vaccine. Acceptance criteria should be determined by performing multiple tests on several lots (preferably three or more) that have shown acceptable performance (i.e. efficacy, immunogenicity, or both) in clinical studies. If geometric mean antibody levels, in absolute terms or relative to the geometric mean antibody
levels induced by the reference vaccine, are below the established limit, or if the $ED_{50}$ or $ED_{50}$ ratio fails to meet the established limit, immunizations and ELISAs should be repeated for those antigens that fail the test. After a second test (if valid), the geometric mean antibody levels, geometric mean ratio, $ED_{50}$ or $ED_{50}$ ratio should be calculated, and results of the two tests may be combined by appropriate statistical methods. The acceptance criteria to consider when two tests are performed should be statistically adjusted. If the results of single or double tests for all antigens in the vaccine satisfy their corresponding limits, the vaccine passes the immunogenicity test. If any antigen does not satisfy its adjusted limit after two assays, the vaccine fails the test.

The test – including the specifications, the method used to calculate antibody response, and the treatment of non-responder mice in the calculation of vaccine potency – should be approved by the NRA.
Appendix 5

Method for respiratory challenge

The respiratory challenge model is designed to demonstrate the protective effect of immunizing mice with acellular pertussis vaccines or candidate antigens. However, it is important to note that the activity of a vaccine or antigen in this model is not an index of clinical efficacy. In general, it involves immunizing mice, which have the capacity to give an adequate immune response to pertussis vaccine, with pertussis vaccine at appropriate doses. Mice are then challenged with live *B. pertussis* suspension. Two challenge routes/methods have been reported, namely intranasal or aerosol administration of challenge. The response to challenge is measured by dissecting out mouse lungs after a suitable time and determining the number of bacterial colony-forming units (CFU). The mouse protective effect of a test vaccine can be determined by comparison of its responses with the responses of a vaccine of known clinical efficacy or an appropriate Reference Preparation. The aerosol challenge method requires specialized aerosol equipment and this may not be readily available in most laboratories. The intranasal challenge method using a harmonized protocol has been proved to be transferable between laboratories in international collaborative studies. However, the current assay is not designed as a routine test for determining vaccine potency. Nevertheless, by comparing with a reference vaccine included in the assay, the respiratory challenge method may be useful to assess the potential impact of changing the manufacturing process and/or formulation; to evaluate new formulations; to investigate potential interactions in combinations; to monitor stability of product and to assess lot consistency. A number of designs are possible with variation in age of the mice, sampling times, and so on. A brief outline of the procedure for a harmonized intranasal/challenge method is given below.

**Mice**

Balb/c mice, three weeks old; 15 mice are to be ordered per vaccine group (i.e. five mice at each time of sampling. Sampling time can be at two hours, five days and eight days post-challenge; or alternatively on other days after the validation study).

**Immunization**

**First immunization:**

Prepare vaccine doses: one vaccine dose = ¼ of a human dose (e.g.125 µl) per mouse and per immunization. The mice are immunized using a 1 ml syringe.
The syringe is divided into 125 µl sections with a marker. The vaccine is injected subcutaneously. When the vaccination is correct, a liquid-filled blister should be visible under the skin.

The second immunization is carried out two weeks after the first immunization, and as described above.

The challenge

All animals are challenged two weeks after the second immunization.

Preparation of challenge suspension:

The bacterial suspension of *B. pertussis* 18323 used for challenge is prepared from an 18–24 hrs culture grown on Bordet–Gengou medium (alternatively, charcoal agar plates containing blood may also be used). Colonies are picked and resuspended in fresh Stainer–Scholte medium or in 1% casamino acids solution. The opacity of the bacterial suspension is adjusted to OD$_{650\text{nm}}$ = 1, which corresponds to 3×10$^9$ CFU/ml (this may vary according to the individual laboratory) and further dilution is carried out to obtain a suspension containing 10$^8$ CFU/ml. Fifty µl of this suspension is used for infection of each mouse.

One aliquot of this suspension is serially diluted to 10$^{-4}$, 10$^{-5}$ and 10$^{-6}$, and 100 µl of the latter two dilutions are plated on Bordet–Gengou plates, in duplicate, in order to enumerate the CFU/ml content of the bacterial suspension used to infect the mice.

Intranasal challenge:

The mice immunized by each vaccine under test should survive until challenge, and each mouse should appear healthy prior to challenge.

All mice are anaesthetized before the challenge. A total of 50 µl of the bacterial suspension is delivered in the nostril, or 25 µl into each nostril, with an automatic 50 µl pipette (in some laboratories, the nose of the mouse is dried with a paper towel before the challenge).

Sampling and CFU count

Five lungs from each group of mice are removed two hours, five and eight days post-challenge following terminal anaesthesia and deposited into tubes containing 1 or 2 ml of saline or 1% casamino acids. The lungs are homogenized individually and plated out under appropriate dilution on a Bordet–Gengou plate or charcoal agar plate. Plates are incubated at 36–37 °C for 4–5 days.

Result

For one animal the lungs homogenate (in 1 ml) is normally diluted to 10$^{-1}$, 10$^{-2}$, 10$^{-3}$ and 10$^{-4}$, and up to 10$^{-6}$ may be needed for the control group.
For each point, the number of colonies on each plate is counted.

Mean of CFU/lungs:

\[ m = \frac{\sum \text{CFU on the 3 plates } \times \text{dilution factor}}{\sum \text{volumes used}} \]

**Note:** dilution factor: 10 if the lungs were homogenized in 1 ml or 20 if the lungs were homogenized in 2 ml.

Example:
For one animal the lungs homogenate (in 1 ml) was diluted 10\(^{-2}\), 10\(^{-3}\) and 10\(^{-4}\):
For 10\(^{-2}\): 500 colonies were counted
For 10\(^{-3}\): 47 colonies were counted
For 10\(^{-4}\): 6 colonies were counted

\[ M = \frac{500 + 47 + 6}{0.01 + 0.001 + 0.0001} \times 10 = 5.53 \times 10^5 \text{ CFU/ml} \]

Log\(_{10}\) is calculated for each mouse and the arithmetic mean is calculated for each vaccine group.

The curve is then traced: mean of log\(_{10}\) CFU/lungs versus day after infection.

**Suggested validity criteria of the test**
Several important criteria have to be met in order to validate the assay.

- Respect of the protocol: there is no technical problem in assay performance.
- The number of bacteria used to challenge the mouse is not below 10\(^5\).
- The calculated log\(_{10}\) mean number of CFU/lung for the negative control group at two hours after challenge should be above 5.4.
- The calculated log\(_{10}\) mean number of CFU/lung for the positive reference group at five days after challenge should be below 3.75.
- A significant difference of at least 3.1 log CFU between the reference group and the negative control group should be detected at five days after challenge.

Both reference and test vaccines should be included in the assay. The log\(_{10}\) CFU/lung for the test and reference vaccines can be used for quantitative analysis, either for comparison of results for groups of mice treated with single doses or, if suitable doses of each vaccine have been used, interpretation of potency using a parallel line assay could be achievable.
Appendix 6

Summary protocol for production and testing of acellular pertussis vaccine

1. Summary information on final lot
Name and address of manufacturer: ________________________________
Lot no.: ________________________________
Date of filling: ________________________________
Date of manufacture: ________________________________
Nature of final product (absorbed): ________________________________
Volume of each recommended single human dose: ________________________________
No. of doses per final container: ________________________________
No. of final containers: ________________________________
Expiry date: ________________________________

2. 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: ________________________________

Cultures media for production
Name of the culture medium: ________________________________

Control of bacterial purity
Result: ________________________________
Date: ________________________________

Control of antigen purification
Purification of PT: ________________________________
Purification of FHA: ________________________________
Purification of pertactin: ________________________________
Purification of FIM 2/3: ________________________________

Identification: ________________________________
Volume: ________________________________
Test on purified antigens

For purified antigens
   Methods: 
   Purity: 
   Date: 

For co-purified antigens
   Purity of claimed antigens: 
   Proportion of each antigen claimed: 
   Methods: 
   Date: 

Residual level of endotoxin
   Methods: 
   Content: 
   Date: 

Antigen content
   Methods: 
   Content: 
   Date: 

Sterility test:
   Tests for bacteria and fungi
      Method: 
      Media: 
      Number of containers tested: 
      Volume of inoculum per container: 
      Volume of medium per container: 
      Temperatures of incubation: 
      Date of test (start, end): 
      Result: 

Detoxification
   Detoxifying reagent: 
   Detoxifying conditions: 

Control of bulk
   Identification: 
   Volume: 
Test for antigen content

Methods: 

Content: 

Date: 

Residual activity of pertussis toxin

1. HIST by temperature measurement

Date: 

Strain of mice/Sex of mice: 

No. of mice per dilution: 

No. of mice dilutions injected: 

Age range or weight range on day of immunization: 

Immunization route/Immunization dose: 

Challenge route/Challenge dose: 

Interval between immunization and challenge: 

Results (IU/SHD or HSU/SHD): 

Calculation method: 

Rectal temperature or dermal temperature: 

<table>
<thead>
<tr>
<th>Temperature</th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference dilution 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reference dilution 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reference dilution n</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test vaccine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Dilution must contain 1.09 or 2.18 IU (0.2 or 0.4 HSU)/SHD.

2. HIST by lethal end-point assay

Date: 

Strain of mice/Sex of mice: 

No. of mice per dilution: 

No. of mice dilutions injected: 

Age range or weight range on day of immunization: 

Immunization route/Immunization dose: 

Challenge route/Challenge dose: 

Interval between immunization and challenge: 

Result: 
<table>
<thead>
<tr>
<th>Reference dilution 1</th>
<th>/</th>
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</thead>
<tbody>
<tr>
<td>Reference dilution 2</td>
<td>/</td>
</tr>
<tr>
<td>Reference dilution n</td>
<td>/</td>
</tr>
<tr>
<td>Test vaccine</td>
<td>/</td>
</tr>
<tr>
<td>Negative</td>
<td>/</td>
</tr>
</tbody>
</table>

**Residual level of endotoxin**

Methods: 
Content: 
Date: 

**Sterility test**

Tests for bacteria and fungi

Method: 
Media: 
Number of containers tested: 
Volume of inoculum per container: 
Volume of medium per container: 
Temperatures of incubation: 
Date of test (start, end): 
Result: 

**Control of final bulk**

Identification: 
Volume: 

**Detoxifying agent**

Methods: 
Content: 
Date: 

**Preservative content**

Methods: 
Content: 
Date: 

**Adjuvant**

Methods: 
Sterility
Tests for bacteria and fungi
Method: ________________________________
Media: ________________________________
Number of containers tested: ________________________________
Volume of inoculum per container: ________________________________
Volume of medium per container: ________________________________
Temperatures of incubation: ________________________________
Date of test (start, end): ________________________________
Result: ________________________________

Residual activity of pertussis toxin
1. HIST by temperature measurement
Date: ________________________________
Strain of mice/Sex of mice: ________________________________
No. of mice per dilution: ________________________________
No. of mice dilutions injected: ________________________________
Age range or weight range on day of immunization: ________________________________
Immunization route/Immunization dose: ________________________________
Challenge route/Challenge dose: ________________________________
Interval between immunization and challenge: ________________________________
Results (IU/SHD or HSU/SHD): ________________________________
Calculation method: ________________________________
Rectal temperature or dermal temperature: ________________________________

<table>
<thead>
<tr>
<th>Reference dilution 1</th>
<th>Temperature</th>
<th>average</th>
<th>variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference dilution 2</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Reference dilution n</td>
<td></td>
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<tr>
<td>Test vaccine</td>
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<tr>
<td>Negative</td>
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<td></td>
</tr>
</tbody>
</table>

- Dilution must contain 1.09 or 2.18 IU (0.2 or 0.4 HSU)/SHD.
2. HIST by lethal end-point assay

Date: 
Strain of mice/Sex of mice: 
No. of mice per dilution: 
No. of mice dilutions injected: 
Age range or weight range on day of immunization: 
Immunization route/Immunization dose: 
Challenge route/Challenge dose: 
Interval between immunization and challenge: 
Result: 

<table>
<thead>
<tr>
<th>No. of deaths/No. of animals inoculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference dilution 1</td>
</tr>
<tr>
<td>Reference dilution 2</td>
</tr>
<tr>
<td>Reference dilution n</td>
</tr>
<tr>
<td>Test vaccine</td>
</tr>
<tr>
<td>Negative</td>
</tr>
</tbody>
</table>

Reversion to toxicity
Incubation period: start date _______ end date _______ temperature _______
Methods: 

1. HIST by temperature measurement

Date: 
Strain of mice/Sex of mice: 
No. of mice per dilution: 
No. of mice dilutions injected: 
Age range or weight range on day of immunization: 
Immunization route/Immunization dose: 
Challenge route/Challenge dose: 
Interval between immunization and challenge: 
Results (IU/SHD or HSU/SHD): 
Calculation method: 
Rectal temperature or dermal temperature:
### Temperature

<table>
<thead>
<tr>
<th></th>
<th>average</th>
<th>variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference dilution 1</td>
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<tr>
<td>Reference dilution 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reference dilution n</td>
<td></td>
<td></td>
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<tr>
<td>Test vaccine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Dilution must contain 1.09 or 2.18 IU (0.2 or 0.4 HSU)/SHD.

### 2. HIST by lethal end-point assay

Date: 
Strain of mice/Sex of mice: 
No. of mice per dilution: 
No. of mice dilutions injected: 
Age range or weight range on day of immunization: 
Immunization route/Immunization dose: 
Challenge route/Challenge dose: 
Interval between immunization and challenge: 
Result: 

<table>
<thead>
<tr>
<th></th>
<th>No. of deaths/No. of animals inoculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference dilution 1</td>
<td>/</td>
</tr>
<tr>
<td>Reference dilution 2</td>
<td>/</td>
</tr>
<tr>
<td>Reference dilution n</td>
<td>/</td>
</tr>
<tr>
<td>Test vaccine</td>
<td>/</td>
</tr>
<tr>
<td>Negative</td>
<td>/</td>
</tr>
</tbody>
</table>

**Immunological activity**

### 1. MIT

Strain of mice: 
No. of mice per dilution: 
No. of dilutions injected: 
Volume and route of injection: 
Identification of reference: 
Date of bleeding: 
Antibody titration: 
Result for test vaccine: ____________________________________________
  GMT value of anti-PT: ____________________________________________
  GMT value of anti-FHA: ____________________________________________
  GMT value of anti-PRN: ____________________________________________
  GMT value of anti-Fims: ____________________________________________
Result for reference vaccine: _________________________________________
  GMT value of anti-PT: ____________________________________________
  GMT value of anti-FHA: ____________________________________________
  GMT value of anti-PRN: ____________________________________________
  GMT value of anti-Fims: ____________________________________________
Or ratio of test vaccine to reference vaccine: ______________________________
  Ratio for anti-PT: _________________________________________________
  Ratio for anti-FHA: _________________________________________________
  Ratio for anti-PRN: _________________________________________________
  Ratio for anti-Fims: _________________________________________________
Date: _____________________________________________________________

2. MICA
Strain of mice: ______________________________________________________
No. of mice per dilution: ______________________________________________
No. of dilutions injected: ______________________________________________
Volume and route of injection: _________________________________________
Date of injection: ____________________________________________________
Identification of reference: ____________________________________________
LD50 in challenge dose: ______________________________________________
No. of colony-forming units in challenge dose: ____________________________
Date of challenge: ____________________________________________________
Date of end of observation: _____________________________________________
Results (IU/SHD): ____________________________________________________
Calculation method: ________________________________________________

<table>
<thead>
<tr>
<th>Dilution</th>
<th>No. of survivors/ No. of animals inoculated</th>
<th>Median effective dose (ED50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaccine: (IU/ml)</td>
<td></td>
<td>ml</td>
</tr>
<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: ____________________________.
**Final product**

Identification: ________________________________
Volume: ________________________________

**Identity test**

Method of testing: ________________________________
Result: ________________________________
Date of test: ________________________________

**Sterility test**

Tests for bacteria and fungi
Method: ________________________________
Media: ________________________________
Number of containers tested: ________________________________
Volume of inoculum per container: ________________________________
Volume of medium per container: ________________________________
Temperatures of incubation: ________________________________
Date of test (start, end): ________________________________
Result: ________________________________

**Test for adjuvant**

Nature and concentration of adjuvant/SHD: ________________________________
Method of testing: ________________________________
Specification: ________________________________
Result: ________________________________
Date of test: ________________________________

**Test for preservative**

Nature and concentration of preservative: ________________________________
Method of testing: ________________________________
Specification: ________________________________
Result: ________________________________
Date of test: ________________________________

**pH**

Method of testing: ________________________________
Specification: ________________________________
Result: ________________________________
Date of test: ________________________________

**Endotoxin test**

Method of testing: ________________________________
Specification: ________________________________
Result: _______________________________________
Date of test: ____________________________________

**Immunological activity**
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 biological activity tests in the “final bulk” section.

**Innocuity test**
Tests in mice
- Date of start of test: ____________________________
- Date of end of test: ____________________________
- No. of animals tested: __________________________
- Route of injection: _____________________________
- Volume and route of injection: __________________
- Observation period: ____________________________
- Results (give details of deaths): ________________

Tests in guinea-pigs
- Date of start of test: ____________________________
- Date of end of test: ____________________________
- No. of animals tested: __________________________
- Route of injection: _____________________________
- Volume and route of injection: __________________
- Observation period: ____________________________
- Results (give details of deaths): ________________

**Inspection of final containers**
- Date of inspection: ____________________________
- Organoleptic characteristics: _________________
- Number of containers inspected: ________________
- Percentage of rejected containers: ______________

**3. Certification by the manufacturer**

Name of head of 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 acellular pertussis vaccine, whose number appears on the label of the final containers, meets all national
requirements and satisfies Part A\textsuperscript{1} of the WHO Recommendations to assure the quality, safety and efficacy of acellular pertussis vaccines (2013)\textsuperscript{2} (if applicable).

Name (typed) ________________________________________
Signature ____________________________________________
Date ________________________________________________

4. Certification by the NRA

If the vaccine is to be exported, attach a certificate from the NRA as shown in Appendix 7, a label from a final container and an instruction leaflet for users.

\textsuperscript{1} With the exception of provisions on distribution and shipping, which the NRA may not be in a position to assess.

Appendix 7

Model certificate for the release of acellular pertussis vaccine by NRAs

This certificate is to be provided by the NRA of the country where the vaccine has been manufactured, on request of the manufacturer.

Lot release certificate

Certificate no. __________________________

The following lot(s) of acellular pertussis vaccine produced by ____________\(^1\) in ____________\(^2\) whose numbers appear on the labels of the final containers, complies with the relevant specification in the marketing authorization and provisions for the release of biological products\(^3\) and Part A\(^4\) of the WHO Recommendations to assure the quality, safety and efficacy of acellular pertussis vaccines (2013)\(^5\) and comply with WHO good manufacturing practices for pharmaceutical products: main principles;\(^6\) Good manufacturing practices for biological products;\(^7\) and Guidelines for independent lot release of vaccines by regulatory authorities.\(^8\)

The release decision is based on ____________________________\(^9\)

The certificate may include the following information:

- Name and address of manufacturer
- Site(s) of manufacturing

---

\(^1\) 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 NRA.
\(^4\) With the exception of provisions on distribution and shipping, which the NRA may not be in a position to assess.
\(^9\) Evaluation of summary protocol, independent laboratory testing, and/or specific procedures laid down in defined document etc. as appropriate.
- Trade name and common name of product
- Marketing authorization number
- Lot number(s) (including sub-lot numbers and packaging lot numbers if necessary)
- Type of container
- Number of doses per container
- Number of containers/lot size
- Date of start of period of validity (e.g. manufacturing date) and/or expiry date
- Storage condition
- Signature and function of the authorized person and authorized agent to issue the certificate
- Date of issue of certificate
- Certificate number.

The Director of the NRA (or other authority as appropriate):

Name (typed) __________________________________________
Signature __________________________________________
Date __________________________________________
Annex 5

Generic protocol for the calibration of seasonal and pandemic influenza antigen working reagents by WHO essential regulatory laboratories

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Abbreviations

CBER       Center for Biologics Evaluation and Research
ERL        Essential regulatory laboratory
GISRS      WHO Global Influenza Surveillance and Response System
NIBSC      National Institute for Biological Standards and Control
NIID       National Institute for Infectious Disease
PLS        Primary liquid standard
SDS–PAGE   Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SRD        Single radial diffusion assay
SRID       Single radial immunodiffusion assay
TGA        Therapeutic Goods Administration
WHO        World Health Organization

1. Introduction

Vaccination is the principal measure for preventing influenza and reducing its impact.\(^1\) Since 1973, WHO has provided formal recommendations for the composition of influenza vaccines on the basis of information provided by the WHO Global Influenza Surveillance and Response System (GISRS).

WHO technical consultations are convened each year in February and September to recommend the viruses for inclusion in influenza vaccines for the northern and southern hemispheres, respectively.\(^2\) For countries in equatorial regions, epidemiological considerations influence which recommendation (northern or southern) individual national and regional authorities consider more appropriate.

High-yield candidate vaccine viruses are developed by collaboration between laboratories involved in developing reassortants and WHO collaborating centres following the strain recommendations. Once developed, these candidate reassortants are sent to WHO collaborating centres for characterization of their antigenic and genetic properties before being released to interested institutions on request. Reference reagents are subsequently developed and standardized by ERLs in collaboration with vaccine manufacturers, and are made available to manufacturers worldwide upon request.

---

This document provides a description of the generic protocol for the calibration of influenza antigen working reagents used by the four WHO ERLs. It represents the consensus of the ERLs on the process of assigning a potency value to a newly established influenza antigen reagent for use in potency testing of inactivated influenza vaccines. An influenza antigen working (or reference) reagent is a preparation of inactivated whole virus that has been freeze-dried and calibrated as outlined in this document.

The calibration process involves the preparation of a primary liquid standard (PLS) and a large batch of freeze-dried antigen by one of the ERLs. The PLS is distributed to all other ERLs for independent calibration by physicochemical means. Samples of the freeze-dried antigen are distributed to the ERLs at the same time and are calibrated against the PLS using the single radial immunodiffusion assay (SRID; also SRD).

2. ERLs

The ERLs are as follows:

- Australia – Therapeutic Goods Administration (TGA)
- Japan – National Institute for Infectious Disease (NIID)
- United Kingdom – National Institute for Biological Standards and Control (NIBSC)
- USA – Center for Biologics Evaluation and Research (CBER).

The participation of all ERLs is assumed, as is current practice, with a minimum of three contributing data for each calibration. The laboratories agree a timeline for completion of all calibration tests, with an expectation that most calibrations will be completed within 15 working days.

The lead ERL is the ERL that produces the freeze-dried antigen reference reagent and sends out materials to the other ERLs for use in calibration (as specified in section 4 below). The lead ERL will inform WHO in a timely manner about the availability of a new reagent and progress of the calibration.

3. Reagents supplied to collaborating ERLs

For each antigen reagent to be calibrated, the following is to be supplied:

- at least 30 ampoules of freeze-dried antigen;
- 10 vials of antiserum (if more than one lot is shipped, 10 vials of each lot);
- a batch, preferably with two aliquots, of a whole virus preparation (i.e. the “PLS”) consisting for example of an in-house live or inactivated preparation or liquid pre-freeze-dried antigen.
The PLS will be characterized by all ERLs with respect to protein content and the proportion of haemagglutinin by physicochemical means to independently determine the haemagglutinin content in micrograms. The PLS serves as the standard against which the secondary freeze-dried antigen reagent is calibrated. The PLS is supplied with a protein concentration estimated by the lead ERL.

4. Internal compliance testing and documentation

The lead ERL performs the tests on the supplied materials prior to distribution, provides test data upon request and supplies interim documentation (e.g. instructions for use). The tests performed by the lead ERL are as follows.

- A protein estimation on the PLS, preferably confirmed by using more than one method with a confirmatory value of ±20% of the estimated value.
- An estimated range of working dilutions of the specific antiserum.
- When possible, SRID assays of the PLS and the freeze-dried antigen using the specific antiserum to confirm their antigenic identity, the estimated potency value of the freeze-dried antigen and a qualitative assessment of SRID zones. If specific antiserum becomes available only after distribution of the PLS and freeze-dried antigen, SRID with a cross-reactive antiserum is performed if possible.

5. Antigen reagent supply to vaccine manufacturers

Upon request, vaccine manufacturers are to be provided with freeze-dried antigen reference reagents as soon as they are available and prior to final calibration. These may be supplied with interim estimated values for use in SRID potency assays. Manufacturers’ data (e.g. comparison of SRID values with manufacturers’ in-house methods for preliminary yield analysis of monovalent bulks) can be supplied to ERLs for review.

6. ERL calibration methodology

6.1 Procedure

Calibration of the PLS: the haemagglutinin content of the PLS is determined by physicochemical methods. Total protein is determined by nitrogen analysis and/or Lowry assay. The percentage haemagglutinin is determined by sodium dodecyl
sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using band densitometry, and the haemagglutinin content of the PLS is expressed in micrograms.

- **Preparation of strain-specific antiserum reagents:** the virus is digested with bromelain to remove haemagglutinin from virus particles. The haemagglutinin missing the transmembrane portion is purified and sheep are immunized multiple times to generate hyperimmune antiserum which is tested for suitability in SRID assays in terms of potency and the quality of the SRID zones produced. The serum is not calibrated but a suitable concentration range for its use is suggested. The reference antiserum is aliquoted and distributed with reference antigen.

- **Preparation of reference antigen:** a reference antigen is prepared on an industrial scale, and is aliquoted and lyophilized. The haemagglutinin content of the reference antigen is assigned by SRID against the PLS using the specific antiserum. The ERLs assign potencies to the PLS and reference antigen independently and the lead ERL collates the results and proposes a final consensus value for agreement by the ERLs. Reagents are distributed as soon as they become available. This process takes several weeks. **Note:** specific antigen standards are required for each candidate vaccine virus strain. Specific antisera are required for each recommended vaccine virus – i.e. antisera are prepared for groups of antigenically similar (“like”) viruses, and are cross-reactive between candidate vaccine viruses covered by a particular recommended virus.

### 6.2 Technical details

- **PLS protein estimation:** this should be performed by a recognized assay (e.g. Lowry or total nitrogen determination), according to the local ERL standard operating procedure or methodology. Assays should include an appropriate protein control (ideally a large batch of common protein standard shared between the ERLs).

- **PLS PAGE assay:** the PLS should be treated as appropriate prior to the PAGE analysis (e.g. reduction or deglycosylation). Assays should be performed according to the local ERL standard operating procedure or methodology, with a minimum of two independent assays, preferably performed by different analysts. Protein bands should be visualized using Coomassie blue-based staining.
- **PAGE band analysis:** analysis of PAGE gels should be performed according to the local ERL standard operating procedure or methodology, recording any parameters that vary from their usual procedures. General guidance for confirmation of accuracy of PAGE band analysis:
  - the ratio haemagglutinin 1: haemagglutinin 2 is approximately 3:2
  - the haemagglutinin content should be between 20% and 50% of total protein
  - the analysis is to be repeated if there is > 20% variation between replicates.

- **SRID assay:** the assay is based on diffusion of virus antigen (e.g. detergent-disrupted virus or vaccine) through an agarose gel containing haemagglutinin-specific antiserum. The square of the diameter of the precipitin ring is proportional to the antigen concentration and a standard curve is used to quantify haemagglutinin in vaccine samples. Preferably, three to six assays should be performed involving more than one operator. Assays should be performed using the local ERL standard operating procedure or methodology. Freeze-dried antigen should be analysed using the PLS as standard antigen on plates containing the appropriate antiserum. The final potency value of the freeze-dried antigen is derived using the mean potency values of all assays.

- **Complete the ERL data sheet:** a sample data sheet is attached to this protocol (Appendix 1). The data sheet is to be sent to the lead ERL. Supplementary data may also be included (Appendix 2).

### 7. Assignment of calibrated potency value by the lead ERL

Data generated by the ERLs (see Appendix 1 and Appendix 2) are collected by the lead ERL and compiled for the final potency value agreement and confirmation. Manufacturers’ data may also be considered, if available. Before the assigned value is made public (e.g. through the ERL website, WHO website or Instructions for Use), the final data sheet and proposed calibration value are sent to all participating ERLs for comment and/or approval. The lead ERL has final authority in assigning a potency value.

### 8. Calibration of secondary and replacement reagents

The antigen reagent that is developed first for a given candidate vaccine virus is calibrated according to the process outlined in sections 2–7 above. When
another antigen reagent for the same candidate vaccine virus is required, either as a replacement to replenish stocks or as an alternative reagent provided by another ERL, it is calibrated against the first antigen reagent to ensure equivalence of reagents and to ease the switch from one lot of reagent to a new one. The process for calibration (cross-calibration) of these types of secondary reagents is abbreviated: calibration uses the antigen reagent that was the first to be developed as the relevant calibrant, and not the PLS. In all cases, calibration is performed using the SRID assay. The exact process for cross-calibration varies between ERLs, and the involvement of more than one laboratory is encouraged – either within the same institution or by using external laboratories (e.g. other ERLs, or national control laboratories with proven experience in influenza vaccine potency testing). If this is not feasible, more than one operator within the calibrating ERL laboratory will need to be engaged in the calibration process.

9. Review of this document

The ERLs will review this document periodically – at least once a year – to ensure that it reflects best practice within ERLs and any updated methodology that may be implemented in the future. Review may take place through electronic means or during meetings between ERL representatives. Any updates of this document will be posted on the GISRS web site.3

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### Appendix 1

**Sample data sheet**

**Date:** ______________

**Reference:** ______________

Primary liquid standard ([ERL]): [virus name] ([reassortant]) (Lot no. [yyy])

<table>
<thead>
<tr>
<th>Protein concentration (µg/ml)</th>
<th>No. of deaths/No. of animals inoculated</th>
<th>Haemagglutinin (HA) content (µgHA/ml)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Antiserum:**

Anti-[virus name] sheep antiserum (Lot no. [zzz], [ERL])

x µl/ml agarose

**Sample:**

Lot no. [yyy], [ERL]

<table>
<thead>
<tr>
<th>Tube no.</th>
<th>Haemagglutinin (HA) content (µgHA/vial)</th>
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<tr>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td></td>
</tr>
<tr>
<td>Standard deviation of the mean (SD)</td>
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<tr>
<td>Coefficient of variation (CV %)</td>
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</tr>
</tbody>
</table>
**Appendix 2**

**Supplementary data sheet**

Calibration of [*virus name*] reference antigen lot [*yyy*] with antiserum lot [*zzz*]

Collaborative study summary results

<table>
<thead>
<tr>
<th></th>
<th>TGA</th>
<th>NIID</th>
<th>CBER</th>
<th>NIBSC</th>
<th>Mean</th>
<th>[n]</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
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<td>CV (%)</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Haemagglutinin (% total protein)</td>
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<tr>
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<td>CV (%)</td>
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<tr>
<td><strong>Reference antigen</strong></td>
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<td>Haemagglutinin (µg/ml)</td>
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<td>CV (%)</td>
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Annex 6

Guidelines for thromboplastins and plasma used to control oral anticoagulant therapy with vitamin K antagonists
Replacement of Annex 3 of WHO Technical Report Series, No. 889

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2. Definitions
3. International Reference Preparations of thromboplastins
4. Preparation of thromboplastins
5. Tests on thromboplastins
   5.1 Response to coumarin-induced coagulation defect
   5.2 Content of haemoglobin and serum
   5.3 Opacity and sediment volume
   5.4 Containers
   5.5 Stability
6. Calibration of prothrombin-time systems
   6.1 Calibration of International Reference Preparations
   6.2 Calibration of secondary standards
   6.3 Calibration of individual batches of thromboplastins
7. Calibration procedure
   7.1 Procedure 1: Calibration of a secondary standard using individual fresh plasma or blood samples
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   7.3 Procedure 3: Local system calibration using certified plasmas
8. The use of calibrated thromboplastins in clinical practice
Authors
Acknowledgements
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   Criteria which may assist clinical laboratories in the choice of a reagent
Appendix 2
   Example of the use of the suggested method for reporting the data for the calibration of any system or a secondary standard of thromboplastin against an International Standard preparation
Appendix 3

Example of the use of the suggested method for reporting the data for the calibration of individual batches of thromboplastin
1. Introduction

Oral anticoagulant drugs derived from 4-hydroxycoumarin (and sometimes from indandiones) are widely used in the treatment and prophylaxis of thrombotic disorders. Coumarin drugs inhibit the biosynthesis of vitamin K-dependent coagulation factors by the liver. For each patient, the dose of these drugs must be adjusted periodically to ensure that an adequate, but not excessive, degree of anticoagulation is achieved. The adjustments are made on the basis of the results of the prothrombin-time or a similar test on the patient’s blood. The test, which requires reagents called thromboplastins, is controlled by the use of calibrated thromboplastins and plasmas.

Various types of thromboplastin are prepared commercially and, in order to be able to interpret the results of the prothrombin-time test, it is essential that each reagent is correctly calibrated. This will ensure that the results of tests with different products and batches are reproducible and can be compared. A procedure for the calibration of thromboplastins using a logarithmic plot of prothrombin times (PTs) has been developed (1) and was described in the report of the forty-eighth meeting of the WHO Expert Committee on Biological Standardization (2). With this procedure, the definition of a calibration parameter called the International Sensitivity Index (ISI) became feasible. It is possible to express prothrombin-time results on a common scale, i.e. the International Normalized Ratio (INR), if the ISI of the thromboplastin used is known.

Many routine laboratories use automated coagulometers for detection of the clotting end-point. There is now substantial evidence that coagulometers can have unpredictable and marked effects on the ISI of thromboplastins (3–6). Because of these effects, some manufacturers provide a “system ISI” for a particular thromboplastin/coagulometer combination. However, this procedure appears to have limitations since variations in the system ISI with the same reagent and coagulometer at different centres have been demonstrated in collaborative studies (7, 8).

In general, the calibration of a given thromboplastin is more precise if performed against an International Reference Preparation1 of similar composition and from the same species (9–11). A system of coexisting International Reference Preparations has been established in which each of these materials is related to the first primary International Reference Preparation – the First WHO International

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1 International reference materials established by the WHO Expert Committee on Biological Standardization have been denoted, variously, as International Reference Preparations, International Reference Reagents and International Standards. These Guidelines refer to all thromboplastin reference materials established by the WHO Expert Committee, independent of the nomenclature. International reference materials so established are by definition “primary” Reference Preparations, secondary Reference Preparations being calibrated in relation to them.
Reference Preparation of thromboplastin (human, combined), coded 67/40 (see Figure A6.1). Two International Reference Preparations of thromboplastin are currently available from the relevant WHO International Laboratory for Biological Standards: the Fourth WHO International Standard for thromboplastin (rabbit, plain) (coded RBT/05) (12) and the Fourth WHO International Standard for thromboplastin (human, recombinant, plain) (coded rTF/09) (13). Other International Reference Preparations have been discontinued. The development of these preparations is described in section 3.

In theory, the ISI/INR system should ensure that the ISI value calculated for a given reagent is independent of the species from which the International Reference Preparation is derived, because all have been directly or indirectly calibrated against the First WHO International Reference Preparation of thromboplastin (human, combined) (coded 67/40). However, this is not always the case; several observations have demonstrated that reagents calibrated against the Second WHO International Reference Preparation of thromboplastin (human, plain) – a material coded BCT/253 (the predecessor of rTF/95) (14) – provide lower INR values than those calibrated against RBT/79 (the predecessor of RBT/90) or OBT/79 (9, 11, 15). The extent of these differences in INR is not usually large enough to cause serious concerns from a practical point of view. The discrepancy is due to calibration errors that persist because the different International Reference Preparations were not checked against each other in the original studies. A new procedure has now been agreed upon: international thromboplastin Reference Preparations, whatever their origin and composition, will be calibrated against all existing International Reference Preparations in order to ensure consistency of results between different routes of calibration (16).

It is recommended that the International Reference Preparation of the same species or composition should be used for calibration of secondary standards, e.g. working standards, by manufacturers and national reference laboratories. Thus, plain rabbit thromboplastins should be calibrated against RBT/05 and plain human thromboplastins against the human recombinant material rTF/09. It has been demonstrated that bovine or rabbit combined thromboplastins can be calibrated with acceptable precision against RBT/05 (17). Thus, it is recommended that bovine or rabbit combined thromboplastins should be calibrated against RBT/05.

The calibration of prothrombin-time systems is not easy. Furthermore, there is considerable variation in results from different laboratories performing the same procedures, as shown by published multicentre calibration studies (9–14, 18–20). In these studies, interlaboratory variation in ISI, expressed as a coefficient of variation, ranged from approximately 1.7% to 8.1%.

The preparation, certification, and use of deep-frozen or lyophilized plasmas for ISI calibration and INR determination has been described as an important adjunct to fresh-plasma ISI calibration (21). The purpose of these
Guidelines, which replace the Requirements published in the forty-eighth report of the WHO Expert Committee on Biological Standardization (2), now discontinued, is to take account of the above-mentioned observations and to describe in detail the technical methods currently in use. Modifications to the methodology may give comparable results, but must be validated against the methodology described in these Guidelines.

How to use these Guidelines

Both manufacturers and clinical laboratories should be informed of the definitions used for the control of oral anticoagulant therapy with vitamin K-antagonists (section 2). Manufacturers of thromboplastins and certified plasmas should be informed of the current Guidelines. These Guidelines contain information for manufacturers of thromboplastins on the methods for calibration of their reagents against International Standards and for calibration of consecutive lots of each type of reagent (section 6). Calibration with International Standards is generally not to be performed by clinical laboratories.

These Guidelines contain information for clinical laboratories on the methods for simplified local-system calibration as described in section 7.3. The use of certified plasmas in clinical laboratories is described in section 7.3.4. Appendix 1 contains criteria for clinical laboratories that may assist with the choice of a commercial thromboplastin reagent or a set of certified plasmas.

2. Definitions

**International Normalized Ratio (INR):** for a given plasma or whole blood specimen from a patient on long-term oral anticoagulant therapy, a value calculated from the prothrombin-time ratio using a prothrombin-time system with a valid ISI according to the formula $\text{INR} = (\text{PT}/\text{MNPT})^{\text{ISI}}$.

**International Sensitivity Index (ISI):** a quantitative measure, in terms of the First WHO International Reference Preparation of thromboplastin (human, combined), coded 67/40, of the responsiveness of a prothrombin-time system to the defect induced by oral anticoagulants (see Appendix 2).

**Mean normal prothrombin time (MNPT):** the geometric mean of the PTs of the healthy adult population. For practical purposes, the geometric mean of the PT calculated from at least 20 fresh samples from healthy individuals, including both sexes, is a reliable approximation of MNPT. It is recommended that individual samples should be collected and tested over at least three working days in order to include inter-assay variation. It is also recommended that each laboratory should determine MNPT using its own prothrombin-time system. Pooled normal plasma (either deep-frozen or freeze-dried) may be suitable if the clotting time obtained is related to the MNPT value and its storage stability is acceptable.
Prothrombin time (PT) (tissue-factor-induced coagulation time): the clotting time of a plasma (or whole blood) sample in the presence of a preparation of thromboplastin and the appropriate amount of calcium ions. The time is reported in seconds (22).

Prothrombin-time ratio (tissue-factor-induced coagulation relative time): the PT obtained with a test plasma or whole blood divided by the MNPT, all times having been determined using the same prothrombin-time system.

Prothrombin-time system: a procedure by which the PT is determined using a specific thromboplastin reagent and a particular method, which may be manual, e.g. a tilt-tube method, or involve the use of an instrument that records the coagulation end-point automatically. The method should be described and the description should include all procedures and equipment used, e.g. the pipettes and test-tubes.

Thromboplastin: a reagent containing tissue factor and coagulant phospholipids. Many commercial thromboplastins are crude extracts prepared from mammalian tissues, in which tissue factor is only a minor component on a weight basis, and which also contain phospholipids. A preparation of a thromboplastin consisting of a tissue extract alone, either with or without added calcium chloride, is termed “plain”. When the preparation contains adsorbed bovine plasma as a source of additional factor V and fibrinogen it is termed “combined”. Thromboplastins may also be grouped into types, according to the tissue source from which they are derived, e.g. human, bovine, rabbit brain or lung, or human placenta. The tissue-factor component of recombinant human thromboplastin reagents is produced in Escherichia coli or insect cells by recombinant DNA techniques and then lipidated in vitro.

Tissue factor: an integral transmembrane protein functioning as a cofactor enhancing the proteolytic activity of factor VIIa towards factor X and factor IX in the blood. Tissue factor needs to be associated with coagulant phospholipids for the full expression of its cofactor function.

3. International Reference Preparations of thromboplastins

International Reference Preparations, International Standards and International Reference Reagents are intended to serve throughout the world as sources of defined biological activity quantitatively expressed in International Units or in terms of a suitable property or characteristic defining the biological activity. These preparations are used to calibrate secondary standards, which include regional, national and manufacturers’ working standards. Normally, working standards are used for routine calibration of individual batches of thromboplastin, and
working standards should have been calibrated with the appropriate International Reference Preparation. If secondary standards are developed using procedures that involve multiple calibration steps, there is a risk that unnecessary variability and discontinuity will occur in relation to the primary International Reference Preparation because of cumulative serial calibration errors.

Current prothrombin-time systems are based on the use of three different species of thromboplastin reagents: human, bovine and rabbit. Originally, the standardization of these thromboplastin reagents likewise involved three different Reference Preparations, one for each of the three species of plain thromboplastin reagents in use (Figure A6.1).

The First WHO International Reference Preparation of thromboplastin (human, combined) (coded 67/40), was established by the WHO Expert Committee on Biological Standardization in 1976 (23). It was a freeze-dried preparation, filled in sealed glass ampoules, and contained a human brain extract to which adsorbed bovine plasma had been added to optimize the content of non-vitamin-K-dependent coagulation factors (i.e. factor V and fibrinogen). Its ISI value was set at 1.0 by definition. In 1983, this preparation was discontinued and replaced by the Second WHO International Reference Preparation of thromboplastin (human, plain) (coded BCT/253), a human brain extract with no added coagulation factors and an assigned ISI value of 1.1 (24). When stocks of BCT/253 became exhausted, a new preparation of human recombinant thromboplastin (coded rTF/95) was established in 1996 as the Third WHO International Standard for thromboplastin (human, recombinant, plain) with an assigned ISI value of 0.94 (18, 25). When stocks of rTF/95 became exhausted, a new preparation of human recombinant thromboplastin (coded rTF/09) was established in 2009 as the Fourth WHO International Standard for thromboplastin (human, recombinant, plain), calibrated against rTF/95 and RBT/05, with an assigned ISI value of 1.082 (13).

The First WHO International Reference Preparation of thromboplastin (bovine, combined) (coded 68/434) was established by the WHO Expert Committee on Biological Standardization in 1978 (26). It was calibrated using the First WHO International Reference Preparation of thromboplastin (human, combined) (67/40). Another material, also calibrated against 67/40, was established as the Second WHO International Reference Preparation of thromboplastin (bovine, combined) (coded OBT/79), in 1983 with an assigned ISI of 1.0 (27). This material (OBT/79), which was derived from bovine brain and combined with factor V and fibrinogen, was used to calibrate thromboplastin materials of bovine origin and combined thromboplastins of whatever origin. When stocks of OBT/79 became exhausted in 2004, it was not replaced by a new International Reference Preparation of bovine origin.
For the calibration of thromboplastins of rabbit origin, the First WHO International Reference Preparation of thromboplastin (rabbit, plain) (coded 70/178), was established in 1978. This material was calibrated against the First WHO International Reference Preparation of thromboplastin (human, combined) (coded 67/40), in an international collaborative study which also included the First WHO International Reference Preparation of thromboplastin (bovine, combined) (26). When stocks of 70/178 became exhausted, the Second WHO International
Reference Preparation of thromboplastin (rabbit, plain) (coded RBT/79), was established in 1982 with an ISI value of 1.4; this was also calibrated against 67/40 (27). The Third WHO International Reference Reagent for thromboplastin (rabbit, plain) (coded RBT/90), obtained from rabbit brain with no added factors, was calibrated against each of the three species of thromboplastins and established by the WHO Expert Committee on Biological Standardization in 1995 with an ISI of 1.0 (28). When stocks of RBT/90 were exhausted, a new preparation of rabbit brain thromboplastin (coded RBT/05) was established as the Fourth WHO International Standard for thromboplastin (rabbit, plain), calibrated against rTF/95 and OBT/79, with an assigned ISI value of 1.15 (12). This material should be used for the calibration of rabbit thromboplastins as well as bovine thromboplastins.

The widespread use of these International Reference Preparations for calibrating secondary standards reflects the value placed on them by the scientific community responsible for the control of thromboplastins. An independent control of a manufacturer’s ISI assignments by a national reference laboratory is also recommended. National control authorities should consider designating an expert laboratory in their country for testing thromboplastin reagents and plasmas used by clinical laboratories to control oral anticoagulant therapy to ensure that they are in accordance with guidelines published by WHO.

The international reference materials for thromboplastins are in the custody of the National Institute for Biological Standards and Control, Potters Bar, England. Samples of these materials are distributed to national reference laboratories or national control laboratories for biological products and, upon payment of handling charges, to other organizations such as manufacturers, universities, research institutes and hospital laboratories. The principle that WHO International Reference Preparations are distributed free of charge to national control authorities for the purpose of the calibration of national standards has been adhered to since the establishment of international biological standardization activities (29).

4. Preparation of thromboplastins

The method of preparation of thromboplastins should be such that there is consistency from batch to batch and that the preparations are suitable for use in the control of oral anticoagulant treatment. The thromboplastins should comply with the specifications outlined in section 5.

Every attempt should be made to use the least contaminated source material possible and to use a manufacturing procedure that prevents further contamination and the growth of organisms during manufacture. Thromboplastins of animal origin should be prepared only from healthy animals. Thromboplastins prepared from bovine brain should be derived only from cattle from countries
that have not reported indigenous cases of bovine spongiform encephalopathy (BSE) and which have a compulsory BSE notification system, compulsory clinical and laboratory verification of suspected cases and a surveillance programme in place (30).

Human brain tissue should not be used because of the risk of transmission of Creutzfeldt–Jakob disease. Thromboplastins derived from human placenta should be prepared from donors in whom there is no evidence of systemic microbiological infection or localized infection and who have been shown to be free from hepatitis B surface antigen, antibodies to human immunodeficiency viruses (HIV-1 and HIV-2) and antibodies to hepatitis C virus.

5. Tests on thromboplastins

Each batch of thromboplastin should satisfy the following criteria.

5.1 Response to coumarin-induced coagulation defect

The response to the coumarin-induced coagulation defect should be measured by the PT obtained using normal and coumarin plasmas. Thromboplastins with a manual ISI between 0.9 and 1.7 are acceptable. However, ISIs towards the lower end of this scale are desirable, since some studies have shown that interlaboratory variation in ISI is greater for high than for low ISI systems (20). It has been suggested that the INR is less accurate when PT is determined with insensitive thromboplastins that have high ISI values (31).

5.2 Content of haemoglobin and serum

To prevent contamination of the product with (activated) clotting factors, the thromboplastin preparation should be free from serum and show no detectable haemoglobin.

5.3 Opacity and sediment volume

The method of manufacture, particularly the method of breaking up the tissue, has a marked effect on the activity, opacity and sediment volume of the thromboplastin. The opacity of preparations intended for use in photoelectric instruments should be suitably low, as specified by the manufacturer.

5.4 Containers

International Reference Preparations for thromboplastins are freeze-dried in sealed glass ampoules (32), but secondary standards may be freeze-dried in ampoules or vials.
5.5 **Stability**

The method of manufacture should be such that the thromboplastin preparations are stable for at least one year. All reagents eventually lose activity when stored at elevated temperatures, and stability should be checked by an accelerated degradation test (33, 34).

Accelerated degradation studies are considered to be only an indicative rather than an absolute guide to the stability of thromboplastins maintained at the storage temperatures recommended by the manufacturer. Lyophilized standard thromboplastins are routinely stored at low temperatures to maintain their stability. A small part of the standard material may be stored at an even lower temperature (“ultra-low temperature stock”). Under the assumption that the rate of degradation is different under the two storage conditions, a comparison of the results of samples of stock kept under the routine storage conditions with those of the ultra-low-temperature stock can be used to assess the stability status of the standard material (35). The stability of the thromboplastins must also be determined for the conditions under which they are stored, i.e. in a real-time stability study (36, 37).

6. **Calibration of prothrombin-time systems**

Four types of calibration should be distinguished:

a. calibration of International Reference Preparations;

b. calibration of secondary standards, e.g. national Reference Preparations and manufacturers’ working standards;

c. calibration of manufacturers’ commercial preparations against the corresponding working standard (“lot-to-lot” calibration);

d. local-system calibration.

In general, the results of calibrations are used by laboratories other than the calibrating laboratories. The clinical laboratories should therefore be aware of the interlaboratory variation in ISI values for the thromboplastin reagent. Type (d) calibration involves the use of deep-frozen or freeze-dried plasmas with assigned INR or prothrombin-time values which are described below. Type (a) and (b) calibrations should be carried out with a large number of fresh plasma or whole blood samples. Several studies suggest that, under certain circumstances, fresh plasmas for type (c) calibrations can be reliably replaced by frozen, freeze-dried, pooled plasma or plasmas artificially depleted of vitamin K-dependent coagulation factors (38–40). Manufacturers should validate this procedure by means of fresh plasmas.

Prothrombin-time systems should be calibrated in terms of the appropriate International Reference Preparation of thromboplastin, and the response to the
coumarin-induced coagulation defect should be defined by the ISI obtained in the calibration procedure. Supplies of International Reference Preparations are limited, and it is not possible to use these materials in routine tests to calibrate each batch of the many thromboplastins produced by different manufacturers. Calibration of individual batches of thromboplastin should be carried out by comparison with a secondary standard, which should be a batch of the same or a similar thromboplastin calibrated against the appropriate International Reference Preparation.

The basis of the thromboplastin calibration model is necessarily an empirical one. While there is good evidence that the calibration relationship defined in a double-logarithmic plot of PTs is usually linear, and that the same line represents data points for both patients and healthy subjects, the possibility of departure from these assumptions cannot be ruled out. Statistical methods to test deviations from the above-mentioned assumption have been described (41, 42). In the case of marked deviation, the assignment of an ISI would not be meaningful. For practical purposes, the assignment of an ISI is acceptable if INRs calculated with the ISI derived from the overall regression line (i.e. for patients plus healthy subjects) do not differ by more than 10%, in the INR range 2–4.5, from INRs calculated with the equation describing the regression line for patients only (see Appendix 2). A difference of 10% is considered as a critical difference according to the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis (SSC/ISTH) Guidelines on preparation, certification, and use of certified plasmas for ISI calibration and INR determination (21).

6.1 **Calibration of International Reference Preparations**

The calibration of the International Reference Preparations for thromboplastins, and their future replacements, should be carried out as part of international multicentre collaborative studies using fresh coumarin, normal plasma and manual techniques. In recent studies for the calibration of replacement International Standards, approximately 20 centres participated. These centres were located in North and South America, Asia, and Europe (12, 13). Each collaborative study for replacement of an International Reference Preparation should include the testing of all existing International Reference Preparations. The ISI assigned to the replacement material should be the mean of the ISIs obtained by calibration with all existing International Reference Preparations (16).

6.2 **Calibration of secondary standards**

Secondary standards of human origin should be calibrated against the current International Standard, i.e. the Fourth WHO International Standard
for thromboplastin (human, recombinant, plain) (coded rTF/09); plain thromboplastins of rabbit brain and rabbit lung should be calibrated against the Fourth WHO International Standard for thromboplastin (rabbit, plain) (coded RBT/05). Thromboplastins of bovine or rabbit brain combined with adsorbed bovine plasma should also be calibrated against RBT/05.

In view of the interlaboratory variation observed in multicentre calibration studies, it is recommended that calibration of national reference materials or manufacturer’s working standards should be performed by at least two laboratories. The ISI assigned to the national reference material or the manufacturer’s working standard should be the mean of ISIs obtained by the individual laboratories.

6.3 **Calibration of individual batches of thromboplastins**

The precision of calibration is greatest when similar materials and methods are compared. For this reason, a national Reference Preparation or manufacturer’s working standard used for the calibration of individual batches of thromboplastin should be a thromboplastin with similar characteristics to the batches being calibrated (i.e. it should be derived from the same tissue of the same species, using a similar manufacturing process). Batch-to-batch calibration should be performed by the manufacturer before release of the reagent, and consistency of ISI values should be shown. Manufacturers should state the applicable end-point detection systems including any relevant coagulometer lines alongside any stated ISI values.

**7. Calibration procedure**

The calibration procedure entails the determination of a series of PTs, using normal and abnormal plasmas or whole blood samples, with both the reference and the test thromboplastin. The tests are performed using either fresh samples from individual subjects (procedure 1) or freeze-dried or frozen plasmas (procedures 2 and 3). Abnormal plasmas for procedure 1 are obtained from patients undergoing long-term oral anticoagulant treatment. Freeze-dried or frozen plasmas for procedure 2 may be pooled plasmas from healthy subjects and from patients undergoing long-term anticoagulant treatment.

Procedure 1 is recommended for the calibration of secondary standards or any other prothrombin-time system against the appropriate International Reference Preparation and for the calibration of whole-blood coagulometers. Procedure 1 can also be used for the calibration of individual batches of thromboplastin against the corresponding secondary standard (i.e. lot-to-lot-calibration), but may be replaced by procedure 2 if the same results are obtained.
The precision of the calibration relationship depends on the number of plasmas and on a balanced distribution of normal and abnormal plasmas over the “therapeutic” range of INR values. The recommended number of abnormal plasmas is three times the number of normal plasmas.

7.1 Procedure 1: Calibration of a secondary standard using individual fresh plasma or blood samples

This procedure consists of a set of tests using freshly opened or reconstituted thromboplastins and a number of different individual samples of fresh plasma or whole blood. The procedure should be repeated on at least five separate occasions using fresh reagents on each occasion (see section 7.1.4). The procedure need not be repeated on consecutive days but should be completed as soon as possible. The tests in any one laboratory on any one day should be performed by the same person.

7.1.1 Blood samples

Blood samples from healthy subjects and patients who have been on oral anticoagulants for at least 6 weeks should be selected. Samples from patients treated with heparin should not be used. Patients’ samples with INR values in the range 1.5–4.5 should be selected.

Blood should be obtained by venipuncture, avoiding haemolysis and contamination with tissue fluids. It should be drawn either with a plastic syringe and transferred to a plastic tube, or with other non-contact activation equipment. Nine volumes of blood should be decalcified with one volume of 109 mmol/l trisodium citrate solution. A mixture of trisodium citrate and citric acid is also acceptable if the total citrate plus citric acid concentration is 109 mmol/l and the pH is no lower than 5. The same procedure and materials should be used for all the samples in a given calibration.

The lot number of tubes used for blood collection should be noted, as there may be lot-to-lot variation. If tubes are made of glass, they must be properly siliconized internally and the pH of the trisodium citrate plus citric acid solution must be in the range 5–6. The sample should be centrifuged as soon as it is received but, in any case, no later than 2 hours after blood collection. The centrifugation should render the plasma poor in platelets (i.e. at least 2500 g for 10 minutes at a controlled room temperature, or at a speed and for a time that allow a platelet count of the platelet-poor plasma lower than \(10 \times 10^9/l\)). The plasma should be taken off the red-cell layer with a plastic pipette, stored undisturbed in a narrow, stoppered, non-contact tube at room temperature and tested within 5 hours after blood collection.

Some techniques or instruments require the use of non-citrated capillary blood. Capillary blood can be obtained by finger or heel puncture. The
capillary blood should be obtained without squeezing of the finger or heel and
tested immediately with the technique or instrument to be calibrated. Venous
blood should be obtained from the same subjects (healthy subjects and patients)
within 5 minutes of taking the capillary sample, for preparation of citrated
plasma as described above and testing with the most appropriate International
Reference Preparation.

7.1.2  Reference thromboplastins
The appropriate International Reference Preparation of thromboplastin (human
or rabbit) should be reconstituted as instructed and the contents of the ampoules
transferred to a container in sufficient volume for all tests to be performed in a
single calibration session. Specific instructions for use should be supplied by the
custodian of these materials.

7.1.3  Prothrombin-time test
The prothrombin-time test is performed either by mixing equal volumes of
citrated plasma, thromboplastin and calcium chloride solution (25 mmol/l), or by
adding a volume of plasma to the required volume of thromboplastin premixed
with calcium, and therefore available as a single reagent. The time (in seconds)
taken for the mixture to clot when maintained at a temperature of between
36.5 °C and 37.5 °C is recorded. Test instructions for commercial thromboplastins
should be provided by the manufacturers.

The coagulation end-point for International Reference Preparations
of thromboplastin must be detected by a manual (tilt-tube) technique because
the manual technique has been used for the establishment of the ISI for the
International Reference Preparations and International Standards. The angle and
speed of tilting the test-tube must be standardized (through 90 °C three times
every 5 seconds) to control glass activation and minimize cooling (45).

The coagulation end-point for other thromboplastins (e.g. commercial
preparations) may be detected with the aid of an automatic or semi-automatic
end-point recorder. The same technique should be used throughout the series of
tests with a given thromboplastin.

Each laboratory should have a system for internal quality control. Records
should be maintained of the lot number of all reagents and disposable equipment
used. Periodic checks of the temperature of incubation baths or heating blocks
and of the volumes of pipettes or pumps should be made and recorded.

An example of results obtained with procedure 1 is provided in
Appendix 2.

7.1.4  Statistical evaluation
The suggested procedure for calculation of the ISI is given in Appendix 2.
Before the final orthogonal regression line for the ISI is calculated, it is important to detect outliers and any samples beyond the therapeutic range. Outliers may result from technical or clerical errors and may strongly influence the estimated ISI. Outliers may be detected as points with a perpendicular distance greater than 3 residual standard deviations from the preliminary orthogonal regression line calculated with all data included (46). It is suggested that outliers be detected and removed in a single step. In the next step any patient samples beyond the therapeutic range (INR < 1.5 or INR > 4.5) should be removed. In this procedure it is important to assess each patient’s INR as the mean INR determined with the International Standard and with the system being calibrated using the ISI obtained after the removal of outliers. Using the INR determined solely with the International Standard could introduce a bias in the orthogonal regression line and should be avoided (47).

It is not necessary to replace the removed outliers and non-therapeutic patient samples with new samples, provided that the number of patient samples remaining is at least 55. In any case, the within-laboratory coefficient of variation of the slope of the orthogonal regression line for normal samples + patient samples should be 3% or less. The number of normal samples should be at least 20 for the calculation of the MNPT. After removal of outliers and non-therapeutic patient samples, the adequacy of the ISI model should be assessed. The ISI model is deemed adequate if the deviation D of the INR calculated with the ISI model from the INR calculated with the International Standard is not greater than 10% (see equation 19 in Appendix 2). If the deviation of the INR calculated with the ISI model is greater than 10%, it is advisable to use a different model according to Tomenson (42).

The sequence of steps in the statistical evaluation is as follows.

1. Calculate preliminary orthogonal regression line (20 normal samples + 60 patient samples).
2. Detect outliers defined as points with a perpendicular distance greater than 3 residual standard deviations from the preliminary line.
3. Remove outliers in one step and recalculate the orthogonal regression line (normal samples + patient samples) and ISI.
4. Calculate each patient’s INR using the PT determined with the International Standard.
5. Calculate each patient’s INR using the PT determined with the system being calibrated and the ISI from step 3.
6. Calculate each patient’s mean INR from steps 4 and 5.
7. Remove patients with mean INR < 1.5 or mean INR > 4.5.
8. Recalculate the orthogonal regression line (normal samples + patient samples) and ISI.
To assess the adequacy of the ISI model, calculate the deviation $D$ of the INR determined with the ISI model from the true INR for INR = 2.0 and for INR = 4.5. If $D < 10\%$, the ISI model is deemed to be adequate. If $D > 10\%$, use Tomenson’s formula for INR calculation (see Appendix 2).

### 7.2 Procedure 2: Calibration of individual batches of thromboplastin

Calibration of individual batches of thromboplastin may be carried out with pooled normal plasmas and pooled coumarin plasmas or plasmas artificially depleted of vitamin K-dependent coagulation factors (38, 39). The number of plasma pools required for precise calibration is, in general, much smaller than the number of fresh individual plasma samples required for procedure 1. The scatter of data points about the regression line is relatively small because the batch to be calibrated is very similar to the working Reference Preparation and/or because the biological variation caused by individual samples is reduced by the pooling of plasmas. It has been reported that lot-to-lot calibration of bovine and rabbit thromboplastins could be performed with as few as three plasma pools (38, 39), but the accuracy of such a simplified procedure may depend on the quality of the pooled plasmas and the thromboplastin being calibrated. It is recommended that any procedure using pooled or artificially depleted plasmas be validated against the fresh plasma procedure (procedure 1).

#### 7.2.1 Pooled plasma

##### 7.2.1.1 Properties of pooled normal plasma

Plasma should be obtained from healthy adults and should comply with the appropriate section of the *Requirements for the collection, processing and quality control of blood, blood components and plasma derivatives* (48). The normal plasmas for pooling should be obtained from at least 20 different donors with an approximately equal number of males and females. Nine volumes of blood should be decalcified with one volume of 109 mmol/l trisodium citrate solution. The packed-cell volume-fraction should be between 0.35 and 0.45.

The final preparation should be platelet-poor plasma, which has been freeze-dried or frozen (at −40 °C or below) in suitable containers. The stability of deep-frozen plasma should be monitored regularly by testing the PT. Thawing of deep-frozen plasma should be done in a water bath at 37 °C for a fixed time depending on the volume in each container. After reconstitution or thawing, the pH should not be lower than 7.3 and should not exceed 7.9, and the plasma should not show any shortening or prolongation of clotting times for at least 2 hours when held at ambient temperature (49). The stability of freeze-dried normal plasma should be checked by accelerated degradation tests. Such plasma
should not show a prolongation of PT of over 5% after storage for 4 weeks at 37 °C. The factor V content should be between 60% (or 60 IU/dl) and 140% (or 140 IU/dl) of the average content of fresh normal plasma (50).

7.2.1.2 Properties of pooled coumarin plasma

Pooled coumarin plasma is obtained from patients who have been on oral anticoagulant therapy for at least 6 weeks. Coumarin plasmas for pooling should be obtained from at least 20 different donors.

Plasma should not be obtained from donors with a history of jaundice or from those with plasma-lipid abnormalities. The collection of plasma, the properties of the final preparation and the stability of the freeze-dried pools are the same as described above for pooled normal plasma.

The INR of the pooled plasma should be stated, as should the thromboplastins used for its assignment. It should be noted that the INR value of a freeze-dried plasma usually depends on the thromboplastin used for its assignment (51–53). At least two different plasma pools, having an INR between 1.5 and 4.5 and with a difference of at least 1.0 in their INRs, in combination with one normal plasma pool are necessary for the calibration procedure.

The factor V content, opacity and citrate concentration for blood decalcification should comply with the requirements for normal plasma (see above).

7.2.1.3 Freedom from infectious agents

The plasma should be shown to be free from hepatitis B surface antigen, antibodies to human immunodeficiency viruses (HIV-1 and HIV-2) and antibodies to hepatitis C virus.

7.2.2 The test

The test should be carried out using the same procedure as described for procedure 1 (see section 7.1.3). An example of the protocol for the recording of the results is given in Appendix 3. The procedure should be repeated on at least four separate occasions (40), with fresh reagents used on each occasion. At least three plasma pools should be used to permit the testing of linearity.

Freeze-dried plasma pools should be reconstituted at least 15 minutes before the actual test. Plasma that has been frozen and subsequently thawed, or reconstituted freeze-dried plasma, should not be centrifuged, and unused reconstituted or thawed material should be discarded after 2 hours.

7.2.3 Statistical evaluation

An orthogonal regression line should be calculated on the basis of the ln PT value of the pooled plasmas. Individual determinations should be entered...
when multiple determinations for each plasma pool are available. Ln PT for
the working reference thromboplastin system is plotted on the vertical axis and
Ln PT for the test batch of thromboplastin on the horizontal axis. Any samples
with a perpendicular distance greater than 3 residual standard deviations from
the regression line should be removed. After removal of such samples, the final
orthogonal regression line is calculated.

To define the ISI of a batch of thromboplastin, a sufficient number of
tests should be carried out to obtain a within-laboratory coefficient of variation
for the slope of the orthogonal regression line of 3% or less. The recommended
procedure for calculation of the ISI is given in Appendix 3.

7.3 Procedure 3: Local system calibration using certified plasmas

Laboratories may calibrate their own local system (i.e. instrument/thromboplastin
combination) using certified plasmas supplied by manufacturers or reference
laboratories. A certified plasma is a deep-frozen or lyophilized plasma with an
assigned PT or INR value. Two procedures using certified plasmas have been
described and are summarized below.

Local test system ISI calibration – this procedure is a modification of the WHO
method for ISI determination. In a set of plasmas, each plasma is assigned a
manual PT value by the manufacturer or reference centre using an International
Standard for thromboplastin. In the local laboratory, the PTs of each plasma are
measured with the local instrument/reagent combination, and the two sets of
PTs are plotted on a log/log plot. The slope of the orthogonal regression line
is used to determine the local ISI (see Appendix 2), which can then be used
for subsequent determination of INRs from the local PTs and MNPT (7, 8,
54–56). An underlying assumption of the WHO orthogonal regression model
is that a single line describes the relationship between log(PT) of abnormal and
normal plasmas. If there is a significant deviation of the two calibration lines
(i.e. abnormal-only and normal/abnormal combined), a correction according to
Tomenson should be applied (42, 57).

“Direct” INR determination – this procedure involves assignment of INR values
to a set of plasmas with the manual method and an international thromboplastin
standard by the manufacturer or reference centre. The PTs of these plasmas
are measured locally using the local instrument/reagent combination, and the
local test system PTs are plotted against the reference INRs on a log/log plot. An
orthogonal regression line is calculated, and the INRs of patients’ plasmas can be
interpolated directly from local PTs using this line, without the need for ISI or
MNPT determination. Although many studies of direct INR determination were
performed with linear rather than orthogonal regression, the latter is preferable
from a theoretical point of view (see section 7.3.2.5) (53, 58–66).
A number of studies have shown that use of either of these procedures can considerably reduce inter-laboratory imprecision in INR determination (8, 59–61, 67, 68). For example, in one study the mean deviation of 95 local systems from the “true” INR was +14.4% with the manufacturers’ ISI, but was reduced to +1.04% with the local ISI (8). In another study, the inter-laboratory coefficient of variation of the INR was reduced from 12% with the manufacturers’ ISI to 6% using direct INR determination with a certified plasma procedure (59).

It should be recognized that there are a number of different ways in which plasmas can be prepared and certified. The following sections describe the various methods of preparation, certification and use, and their advantages and disadvantages.

7.3.1 Preparation of certified plasmas

7.3.1.1 Type of plasma – AVK (from patients on anti-vitamin K therapy) or artificially depleted of prothrombin complex factors (ART)

The intention is for certified plasmas to be as similar as possible to fresh plasmas from patients, thus on theoretical grounds, AVK plasmas might be preferred, although for practical reasons these have to be pooled, rather than individual, donations. In some studies where the two types of plasmas have been compared, AVK plasmas give closer agreement with fresh plasmas and better inter-laboratory agreement than artificially depleted plasmas (51, 69). Artificially depleted plasmas have several advantages over plasmas from patients on oral anticoagulants, including availability of larger volumes, wider selection of PT values across the therapeutic interval, and the possibly reduced risk of virus transmission (70). It can be argued that larger volumes of AVK plasmas could be obtained by pooling donations from patients on anti-vitamin K therapy, but this procedure would make a spectrum of INR values more difficult to obtain because of averaging of individual INRs in such a pool.

The European Concerted Action on Anticoagulation (ECAA) prepared depleted plasmas using artificial depletion of normal human plasma by selective adsorption of vitamin K-dependent clotting factors with barium sulfate to provide a range of values which spanned the therapeutic interval (54). The ECAA found that there is a small difference between the results obtained with ECAA lyophilized artificially depleted plasmas and lyophilized AVK plasmas in ISI value assignment, but both of these lyophilized plasmas differed by a similar amount from a conventional fresh plasma ISI calibration (54). The mean calibration slopes with both types of lyophilized plasma were generally higher than with fresh AVK plasmas but the differences were not great in clinical terms. It should be noted that the ECAA study was performed with one combination of a human brain International Reference Preparation and recombinant thromboplastin and the manual technique, and that the conclusions may not be applicable to all other reagent/instrument combinations.
The reliability of artificially depleted plasmas and AVK plasmas depends on the method of preparation and certification.

7.3.1.2 Method of preparation – frozen or freeze-dried

Although lyophilization seems a simple solution to the difficulties associated with storage and shipment of certified plasmas, there are problems associated with lyophilized materials.

Studies have shown that the INR of fresh plasmas is largely unchanged on freezing, whereas freeze-drying may change the INR significantly, depending on the method of freeze-drying and the thromboplastin/instrument combination used (50, 71–73). Buffering of plasmas shortly after blood collection can reduce but not eliminate changes after freeze-drying. The magnitude of the changes is not the same for all reagents or instruments. The measured INR of lyophilized certified plasmas depends on the thromboplastin reagent and instrument used. The use of RBT/90 presented problems relating to its poor end-point particularly with lyophilized plasmas giving long PTs.

The widespread use of frozen plasmas presents logistical difficulties due to their potential instability, although in some countries frozen certified plasmas have been used with success regarding the reduction of interlaboratory imprecision (63, 67).

Freeze-dried plasmas represent the most practical option for general laboratories and their use has been associated with reduced interlaboratory imprecision in several studies.

7.3.1.3 Citrate concentration

It is well known that citrate concentration can affect the PT, especially of high INR plasmas (74, 75). Furthermore, citrate concentration has a variable effect on the ISI, but the magnitude of the effect is not the same for all reagents and instruments (75–78). The recommended citrate concentration for the collection of blood (1 volume of citrate solution + 9 volumes of blood) for PT is 0.109 mol/l (3.2%), although concentrations in the range 0.105–0.11 mol/l can be accepted (77), and the citrate concentration of certified plasmas should be as close as possible to that in fresh plasma collected in the above anticoagulant (70). Citrate concentrations of 0.129 mol/l (3.8%) should not be used for PT tests.

7.3.1.4 Number of plasmas

The number of plasmas depends on the purpose for which they are used.

Local test system ISI calibration – according to the present Guidelines, to define the ISI of a working thromboplastin, the number of tests carried out should be sufficient to obtain a within-laboratory coefficient of variation (CV) for the slope
of the orthogonal regression line of 3% or less (see section 7.1.4). In an ECAA study of lyophilized artificially depleted and individual AVK plasmas, it has been shown that the requirement for 60 lyophilized abnormal samples for a full WHO calibration can be reduced to 20 if combined with results from seven lyophilized normal plasmas; reductions below this number were associated with decreased precision of the calibration line and hence increased variability of the INR (79). However, the use of pooled AVK plasmas may reduce the scatter of individual plasmas about the line (80), and with pooled plasmas and repeat testing it is possible that a lower number could be used, e.g. acceptable precision has been achieved with six pooled AVK plasmas containing at least 50 patient samples in each pool and two pooled normal plasmas if these were analysed on at least 3 days (40).

“Direct” INR determination – for “direct” INR determination a smaller number of pooled plasmas can be used. Studies have shown improved inter-laboratory variability with as few as six (63), five (53, 60, 61, 65), four (66), three (58), or two (59, 81) plasmas, but considering that one of the plasmas should be a normal plasma and that at least three plasmas are required to define a line, a set of one normal and at least three abnormal plasmas is recommended. One study documented the within-laboratory imprecision of the slope of a calibration line (one normal + three abnormal plasmas): the CV ranged from 0.1% to 4.6% (64). The number of donations in each pool should be at least 10, but higher numbers are preferable to ensure normal levels of factor V.

For both procedures it is important that the abnormal plasmas be chosen to cover the range of 1.5–4.5 INR. The fibrinogen and factor V content should be between 60% and 140% of the average content of fresh normal plasma (50).

7.3.2 Certification (value assignment) of plasmas

Manufacturers or suppliers are responsible for certification, i.e. value assignment to the plasmas.

7.3.2.1 Thromboplastins for certification

WHO standard or European reference thromboplastins should be used directly if possible. Assuming that the certified plasmas are intended for use with the various types and species of thromboplastin, the two types of WHO standard preparations should be used (human and rabbit). If insufficient supplies of WHO or European standards are available, national or secondary standards can be used provided they have been calibrated against the appropriate WHO or European thromboplastin standards in a multicentre study. If the plasmas are intended for use with only one type of thromboplastin (e.g. human), the
appropriate thromboplastin standard preparation should be used. Several studies have shown that the INR value for some lyophilized plasmas obtained with the previous rabbit standard thromboplastin (RBT/90) was greater than the INR obtained with the human and bovine standard preparations (11, 51, 69, 82), especially for artificially depleted plasmas (52). Certified INRs for lyophilized artificially depleted plasmas determined with the ECAA rabbit plain reference thromboplastin were approximately 15% greater than those determined with the recombinant human International Standard and approximately 30% greater than those determined with the bovine combined International Standard (65).

For use with one manufacturer’s thromboplastin reagent only, certification with the manufacturer’s calibrated reagent is acceptable; such “reagent-specific” value assignments have been shown to be reliable in recent collaborative studies (53, 64). The manufacturer’s thromboplastin reagent used for reagent-specific certification of plasmas should be calibrated by at least two independent laboratories using the original WHO procedure (see section 7.1).

Although thromboplastin standards should be used for the assignment of values, the certified plasmas should be tested for suitability with a variety of commercial thromboplastins before release for general use (see section 7.3.3).

7.3.2.2 Number of laboratories
It is recommended that three to five laboratories should be involved in the certification process for each set of plasmas. An individual laboratory’s mean value should differ by no more than ± 10% of the overall mean (in terms of INR) obtained with a given thromboplastin reagent. If the difference is greater than 10%, the divergent individual laboratory’s value should not be used.

7.3.2.3 Manual technique or instruments
The manual tilt-tube method must be used for International Standard preparations, as described in section 7.1.3. Once certified, the plasmas should be tested for their suitability with various reagent/instrument combinations. Where certification of plasmas is done with one manufacturer’s reagent only, an instrument may be used. In this case the reagent/instrument combination must have been calibrated using the original WHO procedure (see section 7.1).

7.3.2.4 Single or multiple values
For the local test system ISI calibration, the actual values of the PTs of the certified plasmas will differ according to the species of the standard thromboplastin used, and therefore PT values must be independently certified for the different species. For the direct INR determination approach, the INR values of the plasmas should theoretically be the same whichever reference thromboplastin reagent is used.
In practice, differences in INRs obtained using different thromboplastins have been observed with some freeze-dried plasmas; results should not be averaged into a single INR if the INRs obtained with individual standard reagents differ by more than 10% from the mean. Large discrepancies between INRs obtained with different thromboplastins may indicate that the plasmas are unsuitable for use with thromboplastins of all types. It should be noted that the manufacturer or supplier of the certified plasmas should clearly specify the set of reagent/instrument combinations with which their materials may be reliably used (83) (see section 7.3.3).

7.3.2.5 Orthogonal regression

Orthogonal regression is used if each coordinate is subject to independent random error of constant variance (41, 84), e.g. PT measurements with two different reagents recorded by the same instrument or operator. Linear regression is used when one of the values is fixed, i.e. essentially without error. The use of certified plasmas does not conform completely to either of these models, but it is important to recognize that apparently “fixed” values of these plasmas are themselves subject to error. Therefore, orthogonal regression should be used for both procedures, i.e. local system ISI calibration and direct INR determination. The equations for orthogonal regression are given in Appendix 2.

7.3.2.6 International reference plasmas

At present there are no established international reference plasmas. Work has begun on the development of reference plasmas for “direct” INR assignment (58, 82). These could then be used for direct certification of batches of commercial plasmas, in the same way as for coagulation factor assays. One difficulty, as mentioned above, is that of preparing lyophilized plasmas with the same properties as fresh plasmas, and it may be that frozen plasmas have to be used. Furthermore, for long-term use, the stability of such reference plasmas would need to be carefully checked. In the meantime, commercial plasmas will continue to have their values assigned as described above.

It should be noted that the validity of lyophilized certified plasmas may be limited to certain combinations of thromboplastins and coagulometers, and may not be generalizable to all other reagent/instrument combinations.

7.3.3 Validation of certified plasmas

Each set or batch of certified plasmas intended for either local test system ISI calibration or direct INR determination must be validated before release (21). The validation should be the responsibility of the manufacturer or supplier who
may seek help from expert laboratories. The validation process should go through the following steps.

1. Ten or more fresh plasmas from patients on long-term oral anticoagulants are selected to represent the full therapeutic range of anticoagulation.
2. The INR of these fresh plasmas is determined with an appropriate International Standard for thromboplastin, and the mean value (INRₐ) is calculated.
3. The INR of the same fresh plasmas is also determined with a variety of commercial reagent/instrument combinations following the certified plasma procedure (either ISI calibration or direct INR determination). The mean value (INRₖ) is calculated.
4. Finally, paired INR values obtained with the International Standard and with the local system are compared to assess their agreement using Bland and Altman’s procedure (85).

If the relative difference between the mean values INRₐ and INRₖ, i.e. \(2(\text{INRₐ} - \text{INRₖ})/(\text{INRₐ} + \text{INRₖ})\), is 0.1 or less, the set of certified plasmas is considered acceptable and may be released for local ISI calibration or direct INR determination. New batches of the same type of preparation should be validated according to the above procedure.

Studies on simplified local calibration with certified plasmas have been published (65, 81), but the value of the studies is limited if the sets of plasmas have not been validated with fresh plasmas from patients as described in this section.

7.3.4 Use of certified plasmas in clinical laboratories

7.3.4.1 Quality assessment

An important use of certified plasmas is to perform internal or external quality assessment, i.e. to determine whether or not corrective action is necessary (83, 86). For quality assessment, a set of three to five certified plasmas with INRs in the range 1.5–4.5 would be required. The INRs of the certified plasmas should be calculated from local PTs and routine ISI, and compared with the certified INR values. If the differences between routine INR and certified INR are greater than 15%, local ISI calibration or direct INR correction should be performed. In addition, the manufacturer of the reagent and certified plasmas should be informed about the discrepant results. Quality assessment with certified plasmas should be performed regularly at intervals of no more than 6 months and should be repeated whenever there is a change in reagent batch or instrument (e.g. servicing, modification, or new instrument). It should be realized that errors
caused by local pre-analytical factors (e.g. divergent citrate concentration or contamination of citrate with divalent cations) cannot be corrected by certified plasma procedures (87).

7.3.4.2 Method for local ISI determination
PTs should be measured in quadruplicate in the same working session, with the local instrument/reagent combination for the full set of normal and abnormal plasmas. Duplicate PT measurements are permitted if the imprecision of the PT system is not greater than 2% CV. It is recommended to repeat the measurements over three sessions or on 3 days to control for day-to-day variation. Mean local PTs should be plotted on the horizontal axis against the certified PT values on the vertical axis (log scales). Tomenson’s test should be performed to test the hypothesis that the mean log(PT) of the certified normal plasmas lies on the same line as the log(PT) of the certified abnormal plasmas (42, 56, 57). If the hypothesis is not confirmed, Tomenson’s correction formula should be applied (42, 56, 57, Appendix 2). Like-to-like comparison should be used wherever possible, i.e. if the local reagent is a human thromboplastin, the certified values should be those determined with a human Reference Reagent. If the INR difference between the routine ISI and the local ISI calibration procedure is greater than 10%, the calibration should be repeated. If the discrepancy persists, the manufacturer or supplier of the local thromboplastin reagent and coagulometer and certified plasmas should be informed. After consultation with the manufacturer of the certified plasmas and, if possible, an expert laboratory, the clinical laboratory should decide which materials and methods should be used for local ISI calibration.

7.3.4.3 Method for direct INR measurements
This method is simpler to use than the one described above as it does not require local ISI and MNPT determinations. PTs should be measured in duplicate with the local instrument/reagent combination for each certified plasma. To allow for day-to-day variation, the measurements should be repeated on at least three different days. Mean PTs should be plotted on the horizontal axis against the certified INR values on the vertical axis (log scales), and an orthogonal regression line derived. Manufacturers of certified plasmas should state for which thromboplastin brands the certified values are valid and provide instructions on how to calculate the calibration line. The INRs of patients’ plasmas should be calculated from the measured PTs. If the INR difference between the routine ISI procedure and the direct determination is greater than 10%, the certified plasma procedure should be repeated. If the discrepancy persists, the manufacturer or supplier of the local thromboplastin reagent and coagulometer and certified plasmas should be informed. After consultation with the manufacturer of the certified plasmas...
and, if possible, an expert laboratory, the clinical laboratory should decide which materials and methods should be used for direct INR measurement.

8. The use of calibrated thromboplastins in clinical practice

It is possible to express prothrombin-time results on a common scale, i.e. the International Normalized Ratio (INR), provided that the ISI of the thromboplastin and the method used are known. The following formula is used:

\[ \text{INR} = \left( \frac{\text{PT}}{\text{MNPT}} \right)^{\text{ISI}} \]

where PT is the patient's PT and MNPT is the mean normal PT determined with the same thromboplastin and method. The use of the INR enables comparisons to be made between results obtained using different thromboplastins and methods. It is a misconception, however, that for an individual patient's plasma the INR will always be identical when determined with different thromboplastins and methods (42, 88). Different thromboplastins vary greatly in their responsiveness to individual vitamin K-dependent clotting factors, i.e. factors II, VII and X, as well as to some factors that are not dependent on vitamin K, e.g. factor V. Discrepancies between INRs determined with different thromboplastins arising from these biological variations and from additional technical errors are therefore not unexpected.

All medical staff and health auxiliaries involved in managing oral anticoagulant treatment should be encouraged to use the INR system. It should be appreciated, however, that this system can be accurate only in the INR range explored by the calibration procedure, i.e. 1.5–4.5.

Manufacturers of commercial reagents should state on the package insert the ISI of the relevant batch of thromboplastin together with the Reference Preparation against which it has been determined and the instrument for which it is valid.

Authors

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References


Appendix 1

Criteria which may assist clinical laboratories in the choice of a reagent

The purpose of this appendix is to provide criteria that are useful for a clinical laboratory to apply when choosing a thromboplastin reagent. These criteria relate to the manufacturer of the reagent providing standard information to the user on the following:

- instrument-specific International Sensitivity Index (ISI) values for the reagent;
- which International Standard has been used for the ISI calibration;
- whether the adequacy of the ISI model has been checked;
- local-system calibration (if an instrument-specific ISI is not available).

The manufacturer’s provision of the following may assist with the choice of a set of certified plasmas:

- information on the International Standards and other thromboplastin reagents that have been used for the certification and the validation of the set of plasmas;
- a statement that the set of certified plasmas has been validated;
- a value of the relative mean International Normalized Ratio (INR) difference obtained in the validation procedure (according to section 7.3.3 of the main text);
- a list of the thromboplastin reagent brands for which the set of plasmas can be used (for both quality assessment and local-system calibration);
- instructions for calculation of local-system ISI or direct INR measurement;
- a spreadsheet for performing the calculations.
Appendix 2

Example of the use of the suggested method for reporting the data for the calibration of any system or a secondary standard of thromboplastin against an International Standard preparation

Thromboplastins:
1. Recombinant human thromboplastin secondary standard
2. Third WHO International Standard for thromboplastin (human, recombinant, plain) (rTF/95) with an established ISI = 0.94.

End-point recording:
1. Automated photoelectric coagulometer for secondary standard

The tests were conducted on five different days (Table 1). On each day, fresh samples from four healthy subjects and 12 patients were tested (plasma samples from healthy subjects are referred to as “normal”). On each day, different subjects were selected. The automated coagulometer and manual determinations were performed more or less simultaneously.

Table 1
Prothrombin times for the calibration of a secondary standard of recombinant human thromboplastin

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Calculations

The International Sensitivity Index of the secondary standard (ISI_w) is obtained by plotting the prothrombin times of the two thromboplastins on logarithmic scales as shown in Figure A6.2, fitting a straight line of the form:

\[ Y = A + BX \] (1)

and estimating the slope B. The recommended method involves estimation of a linear structural relation (also called an “orthogonal regression equation”). With this technique, the slope B can be estimated as follows.

Consider a set of N independent observations \((x_i, y_i)\), where \(i = 1, 2, 3, \ldots, N\); for N paired tests, \(y_i\) represents the natural logarithm of the measured prothrombin time of the International Standard, and \(x_i\) that of the secondary standard. Write \(x_0, y_0\), for the arithmetic means of the N values of \(x_i, y_i\), respectively. Write \(Q_1, Q_2, P\), for the sums of the squares of \((x_i - x_0)\) and \((y_i - y_0)\), respectively, and P for the sum of their products \((x_i - x_0)(y_i - y_0)\). These quantities are all that is necessary for computing a and b, the least-squares estimators for the parameters A and B of equation (1). Now define:

\[ E = (Q_2 - Q_1)^2 + 4P^2 \] (2)

Then

\[ b = (Q_2 - Q_1 + \sqrt{E})/2P \] (3)

and

\[ a = y_0 - bx_0 \] (4)

are the estimators that minimize the sum of the squares of the perpendicular distances of the N points from the line represented by equation (1). The variance of b is given by:

\[ \text{Var}(b) = \{(1 + b^2)P + NbV\}bV/P^2 \] (5)

where V is defined as

\[ V = (Q_2 - bP)/(N - 2) \] (6)

The standard error of b (\(s_b\)) is the square root of Var(b). The coefficient of variation of b is CV(b) = 100 \(\times\) (\(s_b/b\)).

If \(t\) is a deviate from the \(t\)-distribution, with \((N - 2)\) degrees of freedom and at a chosen probability, approximate confidence limits for B can be obtained by setting an interval \(t \times s_b\) on either side of b.

The residual standard deviation is the square root of V. Outlying points should be rejected if their vertical (i.e. perpendicular) distance from the calibration line is greater than 3 \(\times\) \(\sqrt{V}\).
The ISI<sub>W</sub> for the secondary standard is calculated as follows:

\[ \text{ISI}_{W} = \text{ISI}_{IS} \times b \]  

(7)

where ISI<sub>IS</sub> is the ISI of the International Standard.

The prothrombin-time ratio for a given patient (i) with the secondary standard can be estimated according to the equation

\[ R_{w,i} = \exp(x_i - x_n) \]  

(8)

where \( x_n \) is the mean natural logarithm of the prothrombin times of the normals. Likewise, the prothrombin-time ratio with the International Standard can be estimated according to the equation

\[ R_{IS,i} = \exp(y_i - y_n) \]  

(9)

where \( y_n \) is the mean natural logarithm of the prothrombin times of the normals.

If the same linear structural relation is valid for patients and normals it can be shown that the calibration model implies a relationship between prothrombin-time ratios of the form

\[ R_{IS} = (R_w)^b \]  

(10)

where \( R_w \) is the prothrombin-time ratio obtained with the secondary standard, and \( R_{IS} \) is the prothrombin-time ratio for the International Standard. A similar equation can be written for the prothrombin-time ratio of the First WHO International Reference Preparation of thromboplastin (human, combined) coded 67/40:

\[ R_{67/40} = (R_{IS})^{ISI}_{IS} \]  

(11)

Equations (7), (10) and (11) are the base for calculation of the INR according to the ISI calibration model:

\[ \text{INR}_w = (R_w)^{ISI}_{w} \]  

(12)

One of the underlying assumptions of the ISI calibration model is that a single line describes the relationship between logarithms of prothrombin times of both normal and patient plasmas. Thus the line describing the relationship between logarithms of patient prothrombin times should ideally pass through the mean of the logarithms of normal prothrombin times. In the case of marked deviation, the assignment of an ISI would not be meaningful. The natural way to overcome this problem is to introduce a scale parameter and use a model for prothrombin ratios of the form

\[ R_{IS} = e^d \times (R_w)^{b'} \]  

(13)

The above model is referred to as Tomenson’s (1).
Tomenson’s model leads to the following equation for calculation of the corrected INR\(_{w,p}\):

\[
\text{INR}_{IS} = \text{INR}_{w,p} = \{e^d \times (R_w)^b\}^{ISI}_{IS} \tag{14}
\]

Clearly equation (10) is a particular case of equation (13) but the generalized model will also cope with data sets for which the line describing the relationship between logarithms of patient prothrombin times does not pass through the mean of the logarithms of normal prothrombin times. It can be shown that \(d\) in equations (13) and (14) is estimated as

\[
d = a' + b'x_n - y_n \tag{15}
\]

where \(x_n\) and \(y_n\) are the mean natural logarithms of the prothrombin times of normals determined with the secondary standard and the International Standard, and \(a'\) and \(b'\) the intercept and slope of the “orthogonal regression line” calculated using only the patient data.

**Example**

For the full set of data shown in Table 1, the various parameters were calculated according to equations (3), (4), (5), (6), (7) and (15). The results are shown in Table 2. The next step is to detect any outliers. In this example there was one data pair (patient number 22) for which the distance to the line was greater than \(3\times\sqrt{V}\). This data pair was excluded. The parameters calculated for the remaining 79 data pairs are shown in Table 2. The ISI\(_w\) calculated for the remaining 79 data pairs was 4.8% greater than the preliminary ISI calculated with the one outlier included. Each patient’s INR can be calculated in two ways. The first is to calculate INR from the PT measured with the International Standard:

\[
\text{INR}_{IS,i} = (R_{IS,i})^{ISI}_{IS} \tag{16}
\]

The second way of calculating each patient’s INR is by using the PT measured with the secondary standard:

\[
\text{INR}_{w,i} = (R_{w,i})^{ISIw} \tag{17}
\]

Now it is possible to calculate the mean INR\(_{m,i}\) for each patient’s sample:

\[
\text{INR}_{m,i} = (\text{INR}_{IS,i} + \text{INR}_{w,i})/2 \tag{18}
\]

For example, the INR for patient number 43 is 4.32 with the International Standard and 4.70 with the secondary standard. The mean INR is 4.51 which is just at the limit of the therapeutic range. There are no other patients for whom the mean INR is outside the 1.5–4.5 interval.
The relative difference \( D \) between the INR calculated according to equation (12) and equation (14) is given by:

\[
D = 100 \times \left( \exp(ISI_w \times \left( (y_n + (\ln(INR_{IS})/ISI_{IS}) - a')/b' - x_n) - INR_{IS}\right)/INR_{IS} \right) \quad (19)
\]

In this example the orthogonal regression line calculated for 59 patient data pairs did not pass through the mean of the normal data pairs (see Figure 1). The difference \( D \) calculated at \( INR_{IS} = 2 \) is \(-11\%\) and at \( INR_{IS} = 4.5 \) it is \(7.8\%\). It is therefore important to consider the alternative calibration model according to equation (13). By substituting the values from Table 2 in equation (14) the following formula is obtained:

\[
INR_{wp} = \left\{ e^{0.2431} \times (R_w)^{1.2024} \right\}^{0.94} \quad (20)
\]

**Table 2**

| Parameters calculated for the calibration of a secondary standard (see Table 1) |
|---|---|---|
| | 20 normal samples + 60 patient samples (full data set) | 20 normal samples + 59 patient samples (outlier excluded) | 59 patient samples |
| Intercept | –0.7598 | –0.9432 | –0.0386 |
| Slope | 1.4216 | 1.4889 | 1.2024 |
| CV of slope | 3.3\% | 1.6\% | 3.4\% |
| ISI_{IS} | 0.94 | 0.94 | 0.94 |
| ISI_{w} | 1.336 | 1.400 | – |
| \( \sqrt{V} \) | 0.0865 | 0.0396 | 0.0322 |
| \( d \) | – | – | 0.2431 |
| \( y_n \) | 2.602 | 2.602 | 2.602 |
| \( x_n \) | 2.399 | 2.399 | 2.399 |
Figure 1
Log-log plot of prothrombin times for determination of ISI

Reference

Appendix 3

Example of the use of the suggested method for reporting the data for the calibration of individual batches of thromboplastin

Thromboplastins: 
1. Rabbit brain thromboplastin secondary standard
2. New batch of rabbit brain thromboplastin

End-point recording: 
Automated photoelectric coagulometer

Pooled coumarin plasmas: 
lot 960606, 1–5 (deep-frozen)

Pooled normal plasma: 
lot 900423 (deep-frozen)

The International Sensitivity Index (ISI) and mean normal prothrombin time (MNPT) of the rabbit brain thromboplastin secondary standard used with this automated photoelectric coagulometer are 1.31 and 12.7 seconds, respectively.

The tests were conducted in four separate runs. For each run, thromboplastins were freshly reconstituted and deep-frozen plasmas were freshly thawed. Since the secondary standard and the new batch were both timed with the same photoelectric coagulometer, the order in which the two reagents were tested was alternated from one run to the next. This was done to avoid any bias due to possible instability of the thromboplastins and pooled plasmas.

Calculation

The ISI of the new batch (ISI_b) is calculated as ISI_b = ISI_w × b, where b is the slope of the straight line fitted to a double-logarithmic plot of the prothrombin times in Table 1, with the prothrombin times for the secondary standard and the new batch being shown on the vertical and horizontal axes, respectively. The formula for b is given by equation (3) in Appendix 2. The standard error of b is obtained from equation (5) in Appendix 2. The coefficient of variation (%) of b is 100 × (s_b/b).

Example

For the data from Table 1, the calculated residual standard deviation is 0.02482. One pair of determinations for plasma lot no. 960606-5 (run no. 3)
has a perpendicular distance from the line greater than three residual standard deviations. When this pair is excluded, the calculated value for $b$ is 0.9538. The ISI for the secondary standard is given as 1.31. Thus, the ISI for the new batch is estimated as $1.31 \times 0.9538 = 1.25$. The standard error for $b$ is calculated as 0.0130. The coefficient of variation for $b$ is $100 \times (0.0130/0.9538) = 1.36\%$.

Table 1
Prothrombin times (PT) for the calibration of a new batch of rabbit thromboplastin

<table>
<thead>
<tr>
<th>Run no.</th>
<th>Plasma lot no.</th>
<th>Order of testing (within-run)</th>
<th>PT</th>
<th>Order of testing (within-run)</th>
<th>PT</th>
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<tbody>
<tr>
<td>1</td>
<td>900423</td>
<td>1</td>
<td>14.0</td>
<td>7</td>
<td>15.1</td>
</tr>
<tr>
<td></td>
<td>960606-1</td>
<td>2</td>
<td>20.5</td>
<td>8</td>
<td>21.5</td>
</tr>
<tr>
<td></td>
<td>960606-2</td>
<td>3</td>
<td>29.1</td>
<td>9</td>
<td>31.5</td>
</tr>
<tr>
<td></td>
<td>960606-3</td>
<td>4</td>
<td>32.9</td>
<td>10</td>
<td>36.4</td>
</tr>
<tr>
<td></td>
<td>960606-4</td>
<td>5</td>
<td>36.2</td>
<td>11</td>
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<tr>
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<td>39.7</td>
<td>12</td>
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<tr>
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<td>4</td>
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Table 1 continued

<table>
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<th>Plasma lot no.</th>
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<th>Order of testing (within-run)</th>
<th>PT</th>
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<td>37.6</td>
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<td>44.4</td>
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Annex 7

Assessment criteria for national blood regulatory systems

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The following assessment criteria for national blood regulatory systems were adopted by the WHO Expert Committee on Biological Standardization at its sixty-second meeting, held in Geneva from 17–21 October 2011. This annex reflects the collective views of the WHO Blood Regulators Network, and was developed in response to a request from WHO and the International Conference of Drug Regulatory Authorities for an assessment tool to support NRA capacity-building in relation to the regulation of blood and blood products. The tool is intended to help WHO Member States identify gaps and priorities when developing capacity-building programmes, and to support the introduction of regulation of blood products. The establishment of such regulation was recommended in the 2010 World Health Assembly resolution WHA63.12 on the availability, quality and safety of blood products.
Abbreviations

AE  adverse event
AR  adverse reaction
BRN  WHO Blood Regulators Network
GCP  good clinical practice
GDP  good distribution practice
GMP  good manufacturing practice
ICDRA  International Conference of Drug Regulatory Authorities
NCL  national control laboratory
NRA  national regulatory authority
QMS  quality management system
SOP  standard operating procedure
SPC  summary of product characteristics

Glossary

The WHO Expert Committee on Biological Standardization adopted the following definitions for the purpose of this report.

Approval: a decision to authorize marketing of a drug by a national regulatory authority. The mechanism by which a regulatory authority ensures that there is compliance with regulatory requirements and standards that assure quality, safety and efficacy for all blood products and/or processes and establishments involved in collecting blood donations and/or manufacturing blood products. A regulatory approval is generally a precondition for marketing of a blood product.

Associated medical devices: all devices involved in donor testing and/or manufacturing activities.

Associated substances and materials: all substances or materials involved in manufacturing of blood products, including anticoagulants, additive solutions and storage solutions. These materials are regulated as drugs in some jurisdictions.

Blood component:¹ a constituent of blood (erythrocytes, leukocytes, platelets, cryoprecipitate and plasma) that can be prepared by various separation

¹ Stem cells may or may not be included in the scope of the regulatory activity of the competent authority for blood and blood products. Similar criteria for safety, quality and efficacy should be met as for blood and blood components.
methods and under such conditions can be used either directly for therapeutic purposes or for further processing or manufacturing.

**Blood establishment:** any structure, facility or body that is responsible for any aspect of the collection, testing, processing, storage, release and/or distribution of human blood or blood components when intended for transfusion or further industrial manufacturing.

**Blood product:** any therapeutic substance derived from human blood, including whole blood, blood components and plasma-derived medicinal products.

**Core function:** a specific function through which the regulatory system assures the quality, safety and efficacy of blood products.

**Distributor:** any facility that engages in distribution, including storage, importation or exportation of blood products, which may include wholesalers.

**Essential element:** a basic characteristic of a regulatory system as a whole (such as a legal basis for its activities, enforcement power, independence of the regulator from the regulated parties, etc.), which is fundamentally related to the system's ability to effectively ensure the quality, safety and efficacy of blood products.

**Good clinical practice (GCP):** a standard for the design, conduct, performance, monitoring, auditing, recording, analysing and reporting of clinical trials that provides assurance that the data and reported results are credible and accurate, and that the rights, integrity and confidentiality of trial subjects are protected.

**Good distribution practice (GDP):** the part of quality assurance that ensures the quality of a pharmaceutical product is maintained by means of adequate control of all activities that occur throughout the distribution process.

**Good manufacturing practice (GMP):** all elements in the established practice that will collectively lead to final products or services that consistently meet appropriate specifications and compliance with defined regulations.

**Legislation:** a legal instrument of government that defines laws governing a particular subject matter, e.g. regulation of quality, safety and efficacy of medicines. Laws define the roles, rights and obligations of all parties involved in the subject matter in general terms (see also Regulations).

**Licensing:** authorization by the national regulatory authority for the manufacture, importation, exportation or distribution of medical products.

**Manufacturer:** any natural or legal person (structure, facility or body) with responsibility for any aspect of the following activities in relation to blood products: collection, testing, processing, storage, packaging, labelling, release, and/or distribution.

**National regulatory authority (NRA):** national regulatory authorities (also called national medicines regulatory authorities) are legally established bodies that promulgate and enforce medicines regulations.
Plasma-derived medicinal product: any therapeutic product derived from human plasma and produced by an industrial-scale manufacturing process that pools multiple units. Also called plasma derivatives or plasma-derived products.

Quality management system (QMS): a management system that directs and controls an organization with respect to quality, and that ensures that steps, processes, procedures and policies related to quality activities are being followed.

Registration: a procedure under which information regarding the identification, location(s) and scope of activities of all parties involved in manufacturing or supplying a medicinal product and associated medical devices and substances is submitted to the regulatory authority in order to comply with administrative requirements before starting, continuing or amending relevant activities.

Regulations: legislative instruments of government that provide more prescriptive information regarding compliance with relevant legislation. Regulations are specifically designed to provide the legal framework and details necessary to achieve the administrative and technical goals of legislation.

Standard operating procedure (SOP): a prescriptive document that outlines how an activity is carried out.

Sponsor: an individual, company, institution or organization that takes responsibility for the initiation, management and/or financing of a drug submission or clinical trial.

Vigilance: a mechanism of oversight involving an organized system for gathering safety information. This term encompasses pharmacovigilance, haemovigilance and materiovigilance.

Introduction

Blood transfusion is an indispensable, potentially life-saving medical intervention, and blood products such as clotting factors and some immunoglobulins are designated by WHO as essential medicines. However, the inherent risks of blood and the complexity of providing adequate, timely and equitable access to safe blood products require an organized national or regional blood regulatory system. Within that system, a competent blood products regulatory authority assures that appropriate standards are met for production of blood products and monitoring of blood safety. Consequently, as a pillar for the establishment of safe blood programmes globally, WHO has advocated for the establishment and sustenance of strong national regulatory authorities (NRAs) both in developed and developing countries.

In 2010, in resolution WHA63.12, the World Health Assembly expressed its concern about the inequality of access to patients in different parts of the world to blood products, particularly plasma-derived products, which leaves
many without needed transfusions and many of those with severe congenital and acquired disorders without adequate plasma-derived treatments. In this resolution, the World Health Assembly urged Member States:

*to take all the necessary steps to update their national regulations on donor assessment and deferral, the collection, testing, processing, storage, transportation and use of blood products, and operation of regulatory authorities in order to ensure that regulatory control in the area of quality and safety of blood products across the entire transfusion chain meets internationally recognized standards.*

**Purpose and application of the document**

The purpose of this document is to provide a tool to assist capacity-building of national regulatory authorities (NRAs) for the regulation of blood and blood products. Ancillary to the existence of NRAs to regulate activities assuring the provision of safe blood products, there is currently a need to develop criteria defining best practices or attributes of national blood regulatory systems globally for activities related to regulation of blood products. This document provides a description of elements and functions which may support the creation of an appropriate blood regulatory system where none exists so far, and which may also be used as a tool to assess strengths and gaps of established systems. For both developed and developing countries, an assessment tool that reflects international best practices in blood regulation could serve to highlight strengths of the NRA while identifying gaps or areas for future development. In addition, adoption of global criteria by NRAs could promote international convergence of regulations, which can have a beneficial impact on global safety and availability of blood products.

To promote these objectives, this document identifies the essential elements and core regulatory functions that should be present in an effective NRA to assure the quality, safety and efficacy of blood and blood products, as well as associated substances and medical devices including in vitro diagnostics. Additionally, this document provides major criteria, indicators and associated ratings for the essential elements and core functions that are intended to help NRAs assess their performance in the regulation of blood and blood products and prioritize efforts to address any gaps that are identified.

**Scope of the document**

To achieve the aim of an international best practice national blood regulatory framework, a set of integrated general and specific regulatory functions have been identified that are applicable to all aspects of blood product regulation, from the...
collection of source material through to the quality control of the final product, and covering not only blood products but also associated substances and medical devices, including in vitro diagnostics. Section A of this document identifies essential elements that are necessary to establish the legal basis, authority and general characteristics of the NRA. Section B identifies specific core functions of the NRA that are necessary for comprehensive oversight of blood products, related substances and medical devices. It is recognized that the functions may be interdependent and that in some countries the specific functions captured in this document may not be within the scope of one national blood regulatory authority but may be captured by other national authorities or other acceptable mechanisms to achieve compliance with the assessment criteria. Some regulatory functions may be applicable regardless of the intended use of the blood (e.g. for transfusion purposes or for further manufacturing use). However, regulatory structures should be designed in such a way as to avoid fragmentation and uncoordinated delegation.

This document provides the main criteria and indicators for each essential element and core function. The criteria and indicators provide a framework that will identify areas for improvement to governments, particularly in developing countries. A self-assessment or external assessment process using these criteria could also serve as a useful means to highlight strengths of NRA programmes for regulation of blood products while identifying gaps or areas for future development. National authorities are encouraged to use the assessment criteria as a roadmap towards evolving a best practice blood regulatory system.

It is recognized that many national blood regulatory systems will not be able to meet all the criteria and indicators listed in this document. The criteria and indicators are therefore organized into those considered as being required (R) and thus necessary in order to be effective as a blood regulator, and those that are considered as being desirable or suggested (S) to achieve a blood regulatory system of international best practice.

It is also recognized that single required criteria may not formally be fulfilled even by regulators with proven effectiveness, but that underlying relevant safety issues can be met by other means. This offers the opportunity to compare different ways of ensuring the safety of blood products and to highlight areas where refinement of the assessment criteria may need to be considered.

With experience of their application, future versions of these assessment criteria are expected to better accommodate effective alternatives, and to highlight aspects, such as the prioritization of efforts, that may require additional guidance.
Part A. Essential elements

1. National regulatory system

Applicable to blood, blood components, plasma-derived medicinal products, associated substances, and medical devices including in vitro diagnostics

<table>
<thead>
<tr>
<th>Main criteria related to the element</th>
<th>Rating</th>
<th>Indicators related to the main criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 A comprehensive legal (statutory) basis exists for establishment of a regulatory system applicable to blood, blood components, plasma-derived products, associated substances, and medical devices including in vitro diagnostics.</td>
<td>R</td>
<td>1.1.1 Provisions for the main regulatory functions can be identified and are up to date.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.1.2 The regulations or their adaptations take into consideration developments in the field of blood and related technologies.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.1.3 Regulations have been established and are available; they are intelligible to those that need to comply with or enforce them, and the means of communication used are adequate.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.1.4 Legislation exists that defines therapeutic products for human use to be regulated, and establishes standards of quality, safety and efficacy for: a. blood, blood components and plasma-derived products; b. associated substances and medical devices including in vitro diagnostics.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.1.5 Legislation exists that provides a legal basis for the responsible NRA to perform the essential functions.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.1.6 Legislation enables the appropriate institutions to issue regulations.</td>
</tr>
</tbody>
</table>
### Table continued

<table>
<thead>
<tr>
<th>Main criteria related to the element</th>
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<tbody>
<tr>
<td></td>
<td>Main criteria</td>
<td>Indicator</td>
</tr>
<tr>
<td>S 1.1.7 The development of regulations includes the opportunity for public consultation.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.2 The legislation assigns the enforcement of regulations regarding the products covered in 1.1 to one or more responsible regulatory authorities.</td>
<td>R R 1.2.1 The competent authorities involved in the regulatory system for blood, blood components, plasma-derived products, associated substances, and medical devices including in vitro diagnostics are clearly identified and can be named for each of the regulatory functions.</td>
<td></td>
</tr>
<tr>
<td>R 1.2.2 The responsibilities, functions and the organization of each of these authorities are clearly defined, in particular as regards the scope of the regulation (regulatory functions) they have under their control.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R 1.2.3 The activities of the various authorities involved are coordinated and supervised by an administrative mechanism.</td>
<td></td>
<td></td>
</tr>
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* R = required; S = suggested.
### 2. National regulatory authority

*Applicable to blood, blood components, plasma-derived medicinal products, associated substances, and medical devices including in vitro diagnostics*

<table>
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<tbody>
<tr>
<td></td>
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</tr>
<tr>
<td>2.1 There is independence of the regulatory authority in decision-making.</td>
<td>R</td>
<td>R 2.1.1 A clear division of roles and responsibilities is implemented between the NRA, blood establishments, manufacturers and distributors, reflecting independence of the regulatory system.</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>R 2.1.2 Accountabilities for decision-making are clear.</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>R 2.1.3 An internal policy on potential conflicts of interest for staff exists.</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>R 2.1.4 NRA management and assessment activities (including use of expert committees) never include representatives from manufacturers or licence holders.</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>R 2.1.5 A code of conduct for regulatory staff exists.</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>S 2.1.6 Written procedures for meetings with manufacturers, distributors and other sponsors exist.</td>
</tr>
<tr>
<td>2.2 The NRA has established an institutional development plan.</td>
<td>S</td>
<td>S 2.2.1 The NRA has an institutional development plan, which is implemented and updated.</td>
</tr>
<tr>
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<td>Rating</td>
<td>Indicators related to the main criteria</td>
</tr>
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</tr>
<tr>
<td>S 2.2.2 The development plan includes: vision; strategic objectives; timeline and deadline for target implementation; indicators; functions and/or duties of the NRA; ongoing staff training plan; resources needed; information and/or communication strategy; and a human resources development plan.</td>
<td>S 2.2.3 Performance indicators are established and used for monitoring attainment of objectives.</td>
<td></td>
</tr>
<tr>
<td>2.3 The NRA has adequate resources to carry out its functions properly and to enforce regulatory functions.</td>
<td>R 2.3.1 An adequate number of trained staff and budgetary provisions exist for all essential functions.</td>
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</tr>
<tr>
<td></td>
<td>R 2.3.2 All staff members have appropriate qualifications to conduct regulatory activities and are provided with timely, relevant and regularly updated training.</td>
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<tr>
<td></td>
<td>R 2.3.3 Tasks and responsibilities of staff members are well defined.</td>
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<tr>
<td></td>
<td>R 2.3.4 Mechanisms are in place to ensure that those performing regulatory functions have sufficient and current expertise in specialized areas.</td>
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### Table continued

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<thead>
<tr>
<th>Main criteria related to the element</th>
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<th>Indicators related to the main criteria</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>R 2.3.5 Policies and procedures exist for recruitment and selection of external experts and the management of expert advisory committees, including potential conflict of interest.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R 2.3.6 An agreement between the NRA and external experts defining roles and responsibilities is established.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S 2.3.7 The sources of funding of the responsible authorities performing regulatory functions are defined.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S 2.3.8 Written criteria for selection and recruitment of regulatory staff are defined.</td>
</tr>
<tr>
<td>2.4 A QMS is in place.</td>
<td>S</td>
<td>S 2.4.1 A QMS is implemented by the NRA for all its core functions as specified below.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S 2.4.2 Budgetary provisions are made for implementation and maintenance of the QMS.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S 2.4.3 A qualified quality manager is designated as responsible for the implementation of the QMS.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S 2.4.4 The documentation needed to establish, implement and maintain the QMS is defined (quality manual, SOPs, etc.).</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S 2.4.5 The QMS is based on recognized international standards.</td>
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</table>
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<thead>
<tr>
<th>Main criteria related to the element</th>
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<tbody>
<tr>
<td></td>
<td>Main criteria</td>
<td>Indicator</td>
</tr>
<tr>
<td>2.4.6 The QMS is certified or accredited by external bodies.</td>
<td>S</td>
<td>2.4.6</td>
</tr>
<tr>
<td>2.4.7 An internal and external audit and review system exists as well as evidence that corrective and preventive actions are taken as a result of monitoring and/or audits.</td>
<td>S</td>
<td>2.4.7</td>
</tr>
<tr>
<td>2.5 Transparency and accountability is ensured.</td>
<td>R</td>
<td>2.5.1 Legally-specified, confidential and trade secret information is available for internal use and decision-making. However, all other information is publicly available and kept up to date.</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>2.5.2 Listing of authorized products and companies is made available where needed.</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>2.5.3 Information on sanctions, recalls and public health warnings is publicly available.</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>2.5.4 Information on decisions is available and easily accessible to the public and includes negative decisions in selected cases (may vary depending on national regulation).</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>2.5.5 An opportunity for interaction between the NRA and stakeholders is given.</td>
</tr>
</tbody>
</table>

* R = required; S = suggested.
### Part B. Core functions

#### 3. Licensing and/or registration of blood establishments

*Applicable to blood and blood components including plasma for fractionation*

<table>
<thead>
<tr>
<th>Main criteria related to the function</th>
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<th>Indicators related to the main criteria</th>
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<tbody>
<tr>
<td></td>
<td></td>
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</tr>
<tr>
<td>3.1 Legislative authority exists to require registration and/or licensing of blood establishments, and for enforcement power.</td>
<td>R R 3.1.1</td>
<td>Legislation and/or regulations exist that require a blood establishment that intends to collect, test, process, store, manufacture, distribute, import or export blood and blood components to be authorized, accredited, registered or licensed by the designated NRA.</td>
</tr>
<tr>
<td></td>
<td>R 3.1.2</td>
<td>The NRA has the authority to take regulatory action (e.g. revoke or suspend the licence) if the establishment does not comply with regulatory requirements.</td>
</tr>
<tr>
<td>3.2 A licensing and/or registration system is established and operational for blood establishments.</td>
<td>R R 3.2.1</td>
<td>Activities that are decentralized or delegated to other agencies or authorities follow the standards, guidelines and procedures as agreed by the NRA, and a reporting mechanism is established between the responsible authorities.</td>
</tr>
<tr>
<td></td>
<td>R 3.2.2</td>
<td>Required registration and/or licence applications are assessed by the NRA based on written guidelines.</td>
</tr>
<tr>
<td></td>
<td>R 3.2.3</td>
<td>A list of all licensed and/or registered blood establishments is maintained and made available where needed.</td>
</tr>
</tbody>
</table>
### Table continued

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<thead>
<tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>R 3.2.4 Advice for applicants is available on the content, format, requirements and procedures to follow in order to submit a required registration and/or application for an establishment licence.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S 3.2.5 Facility documentation (e.g. site master file, qualification of a responsible person) is submitted as part of a required registration and/or application for an establishment licence and is assessed to demonstrate that the facility is suitable for the activities to be performed (e.g. blood collection, donor screening, testing, storage, etc.).</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S 3.2.6 Renewal periods for an establishment licence and/or registration are defined and consistent with mechanisms of surveillance.</td>
</tr>
<tr>
<td>3.3 Significant changes to an establishment licence and/or registration are submitted and assessed by the NRA prior to implementation.</td>
<td></td>
<td>R 3.3.1 Changes are assessed based on the type of change.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S 3.3.2 Written guidelines for applicants are available that define the types and scopes of changes and documentation required.</td>
</tr>
<tr>
<td>3.4 Compliance with the principles of good manufacturing practice (GMP) is assessed as part of the establishment licensing and/or registration process.</td>
<td></td>
<td>R 3.4.1 Compliance with applicable principles of GMP is a condition for maintaining an establishment licence and/or registration and for approval of significant changes.</td>
</tr>
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<table>
<thead>
<tr>
<th>Main criteria related to the function</th>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.5 QMS requirements are established for all functions performed by blood establishments.</td>
<td>R</td>
<td>R 3.5.1 The essential components for a QMS are covered.</td>
</tr>
<tr>
<td>3.6 Assessment of compliance with standards regarding donor selection criteria and testing of donations is part of the establishment licensing and/or registration process (alternatively this requirement can be met under core function 5).</td>
<td>R</td>
<td>R 3.6.1 Compliance with national standards is a condition for maintaining an establishment licence.</td>
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<tr>
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<tr>
<td></td>
<td>Main criteria</td>
<td>Indicator</td>
</tr>
<tr>
<td><strong>R 3.6.3</strong> Inspections are carried out for checking compliance with these national standards.</td>
<td>R</td>
<td>3.6.3</td>
</tr>
<tr>
<td><strong>R 3.6.4</strong> Defined procedures are in place for taking action in instances of any nonconformity.</td>
<td>R</td>
<td>3.6.4</td>
</tr>
</tbody>
</table>

* R = required; S = suggested

### 4. Licensing and/or registration of manufacturers and distributors of plasma-derived medicinal products

*Applicable to plasma-derived medicinal products*

<table>
<thead>
<tr>
<th>Main criteria related to the function</th>
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<th>Indicators related to the main criteria</th>
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<tbody>
<tr>
<td></td>
<td>Main criteria</td>
<td>Indicator</td>
</tr>
<tr>
<td><strong>4.1 Legislative authority exists to require registration and/or licensing of manufacturers and distributors of plasma-derived products, and for enforcement power.</strong></td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td><strong>4.1.1</strong> Legislation and/or regulations exist that require manufacturers and distributors of plasma-derived products that intend to manufacture, distribute, import or export plasma-derived products to be registered and/or licensed by the designated NRA.</td>
<td>R</td>
<td>4.1.1</td>
</tr>
<tr>
<td><strong>4.1.2</strong> The NRA has authority to take regulatory action (e.g. revoke or suspend the licence) if the company does not comply with regulatory requirements.</td>
<td>R</td>
<td>4.1.2</td>
</tr>
</tbody>
</table>
### Table continued

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<tbody>
<tr>
<td></td>
<td>Main criteria</td>
<td>Indicator</td>
</tr>
<tr>
<td>4.2 A licensing and/or registration system is established and operational for manufacturers and distributors of plasma-derived products.</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>4.2.1</td>
<td>Activities that are decentralized or delegated to other agencies or authorities follow the standards, guidelines and procedures as agreed by the NRA, and a reporting mechanism is established between the responsible authorities.</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>4.2.2</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>4.2.3</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>4.2.4</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>4.2.5</td>
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<tr>
<td>Main criteria related to the function</td>
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<td>Indicators related to the main criteria</td>
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<tr>
<td></td>
<td>Main criteria</td>
<td>Indicator</td>
</tr>
<tr>
<td>S 4.2.6 Renewal periods for an establishment licence and/or registration are defined and consistent with mechanisms of surveillance.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.3 Significant changes to an establishment licence and/or registration are submitted and assessed by the NRA prior to implementation.</td>
<td>R R 4.3.1 Changes are assessed based on the type of change.</td>
<td>S 4.3.2 Written guidelines for applicants are available that define the types and scopes of changes and documentation required.</td>
</tr>
<tr>
<td>4.4 Compliance with principles of GMP and GDP is assessed as part of the establishment licensing and/or registration process.</td>
<td>R R 4.4.1 Compliance with applicable principles of GMP and GDP is a condition for maintaining an establishment licence and/or registration and for approval of significant changes.</td>
<td>R 4.4.2 National GMP and GDP standards are published and are consistent with or based on recognized standards for the manufacturing and distribution of plasma-derived products.</td>
</tr>
</tbody>
</table>
### Table continued

<table>
<thead>
<tr>
<th>Main criteria related to the function</th>
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<th>Indicators related to the main criteria</th>
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<tbody>
<tr>
<td></td>
<td><strong>Main criteria</strong></td>
<td><strong>Indicator</strong></td>
</tr>
<tr>
<td><strong>R 4.4.3</strong> Periodic inspections according to GMP and GDP principles are carried out for supervision of manufacturers and distributors of plasma-derived products. For inspections carried out abroad: a. there is an agreement with other NRAs for exchange of inspection reports and/or certificates, or b. a list of reference countries and/or agencies whose certificates and decisions are accepted exists, or c. site inspections are carried out abroad.</td>
<td><strong>R</strong></td>
<td></td>
</tr>
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* **R** = required; **S** = suggested.

### 5. Approval of blood and blood components (product and/or process approval)

**Applicable to blood and blood components including plasma for fractionation**

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<thead>
<tr>
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<tbody>
<tr>
<td></td>
<td><strong>Main criteria</strong></td>
<td><strong>Indicator</strong></td>
</tr>
<tr>
<td><strong>R 5.1.1</strong> An approval system is required that includes any imported products.</td>
<td><strong>R</strong></td>
<td></td>
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<tr>
<th>Main criteria related to the function</th>
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<tr>
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<tr>
<td>5.1.2 The NRA has the authority to</td>
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<tr>
<td>issue an approval, to suspend it and</td>
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<td>to withdraw it if the product is</td>
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<td>considered unsafe or does not comply</td>
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<tr>
<td>with regulatory requirements</td>
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<td></td>
</tr>
<tr>
<td>R 5.2.1 The capability exists to</td>
<td>R</td>
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<tr>
<td>perform science-based risk assessments</td>
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<td></td>
</tr>
<tr>
<td>and risk management.</td>
<td></td>
<td></td>
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<tr>
<td>R 5.2.2 Specifications related to</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>quality, safety and efficacy of blood</td>
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<td></td>
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<tr>
<td>and blood components are defined and</td>
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<tr>
<td>under the supervision of the NRA.</td>
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<tr>
<td>R 5.2.3 The critical standards for</td>
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<tr>
<td>product manufacturing are legally</td>
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<tr>
<td>binding and include donor selection,</td>
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<tr>
<td>laboratory testing, component</td>
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<tr>
<td>preparation, storage, issuance,</td>
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<tr>
<td>tracking, tracing, record-keeping,</td>
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<tr>
<td>and safe disposal of units not</td>
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<tr>
<td>meeting specifications for use in</td>
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</tr>
<tr>
<td>transfusion.</td>
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<tr>
<td>S 5.2.4 Procedures to recognize</td>
<td>S</td>
<td></td>
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<tr>
<td>exceptions are clearly defined (e.g.</td>
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<td></td>
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<tr>
<td>if collected by a medical practitioner</td>
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<tr>
<td>for a specific therapeutic purpose).</td>
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<tr>
<td>S 5.2.5 Requirements and standards</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>are based on internationally</td>
<td></td>
<td></td>
</tr>
<tr>
<td>recognized standards.</td>
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</tr>
<tr>
<td>R 5.2.6 Plasma for fractionation</td>
<td>R</td>
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<tr>
<td>meets internationally recognized</td>
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<tr>
<td>standards.</td>
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5.2 A system for ensuring quality, safety and efficacy of blood and blood components is established and operational.
### Main criteria related to the function

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<tr>
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<th>Main criteria</th>
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#### 5.3 Donor selection and deferral criteria are established as appropriate to the intended use of the component.

- **R 5.3.1** Donor selection and deferral criteria (temporary and permanent deferrals) take into account the health of the donor and the safety and suitability of the donation consistent with current science.

- **R 5.3.2** Mechanisms for regularly reviewing and updating the donor selection and deferral criteria are in place and take into consideration the development of issues that might have a negative impact on the quality and safety of blood and blood components, e.g. epidemiological situation or emerging diseases.

#### 5.4 Transmissible disease testing requirements are established as appropriate to the intended use of the component.

- **R 5.4.1** Mechanisms for regularly reviewing (e.g. by qualified experts in epidemiology) and updating the testing requirements are in place.

- **R 5.4.2** Epidemiological data regarding the prevalence and incidence of infectious disease markers in blood donors are available and regularly updated.

#### 5.5 Labelling requirements are established.

- **R 5.5.1** Each blood component has a unique and clear identifier and is fully traceable.

- **R 5.5.2** Original labelling and significant amendments are submitted to the NRA and assessed prior to implementation.
<table>
<thead>
<tr>
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<tbody>
<tr>
<td></td>
<td>Main criteria</td>
<td>Indicator</td>
</tr>
<tr>
<td>S 5.5.3 Product labelling includes information on the risks and benefits of product use.</td>
<td>Rating</td>
<td>S 5.5.4 Requirements are based on internationally recognized standards.</td>
</tr>
<tr>
<td>R 5.6.1 Assessment exists that includes relevant aspects of quality, safety and, where applicable, efficacy of blood and blood components.</td>
<td>R 5.6.2 Guidelines for applicants exist on the content, format and procedures to follow in order to submit an application for approval.</td>
<td>S 5.6.3 Written guidelines for assessment of applications are implemented.</td>
</tr>
<tr>
<td>S 5.6.4 Appeal procedures are in place.</td>
<td>S 5.6.5 An assessment report is prepared and used as a reference for decision-making.</td>
<td></td>
</tr>
<tr>
<td>S 5.7.1 Written guidelines for applicants are available that define the types and scopes of changes and documentation required.</td>
<td>S 5.7.2 Written guidelines for assessment exist based on the type of change (e.g. significant, notifiable, administrative).</td>
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<td></td>
<td>Main criteria</td>
<td>Indicator</td>
</tr>
<tr>
<td>5.8 Appropriate assessment expertise is available.</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>5.8.1 Access to experts with relevant qualifications and experience (internal and/or external) is assured for assessment of blood and blood components (preclinical, clinical and quality data).</td>
<td>S</td>
<td></td>
</tr>
</tbody>
</table>

* R = required; S = suggested.

**6. Approval of plasma-derived medicinal products**

*Applicable to plasma-derived medicinal products*

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<tr>
<td></td>
<td>Main criteria</td>
<td>Indicator</td>
</tr>
<tr>
<td>6.1 Legal provision for a marketing approval system exists to ensure the quality, safety and efficacy of plasma-derived products.</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>6.1.1 Marketing approval is required for plasma-derived products, including imported products.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.1.2 The NRA has the authority to issue marketing approval for plasma-derived products, to suspend an approval, and to withdraw it if the product is considered unsafe or does not comply with regulatory requirements.</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td>6.2 A marketing approval system for plasma-derived products is established and operational.</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>6.2.1 The capability exists to perform science-based risk assessments and risk management.</td>
<td></td>
<td></td>
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<tr>
<td>Main criteria related to the function</td>
<td>Rating*</td>
<td>Indicators related to the main criteria</td>
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<td>----------------------------------------</td>
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<tr>
<td></td>
<td>R 6.2.2</td>
<td>There is a requirement for the applicant to include a list of all the blood establishments that collected the plasma used in the product.</td>
</tr>
<tr>
<td></td>
<td>R 6.2.3</td>
<td>Specifications related to the quality and safety of plasma for fractionation are defined and under the supervision of the NRA.</td>
</tr>
<tr>
<td></td>
<td>R 6.2.4</td>
<td>Selection, deferral and transmissible disease testing requirements for plasma donors are established (see criteria 5.3 and 5.4).</td>
</tr>
<tr>
<td></td>
<td>R 6.2.5</td>
<td>Advice for applicants is available on the content, format and procedures to follow in order to submit an application for market authorization.</td>
</tr>
<tr>
<td></td>
<td>S 6.2.6</td>
<td>Appeal procedures are in place.</td>
</tr>
<tr>
<td></td>
<td>S 6.2.7</td>
<td>The NCL is involved in assessment as appropriate.</td>
</tr>
<tr>
<td></td>
<td>S 6.2.8</td>
<td>Written procedures for selection, management, and use of external experts are available.</td>
</tr>
</tbody>
</table>

6.3 Assessment of applications for market authorization is implemented.

| R 6.3.1 | Assessment of quality, safety and efficacy of plasma-derived products is performed, including assessment of the effectiveness of measures used by manufacturers to inactivate and/or remove transmissible pathogens. |
Table continued

<table>
<thead>
<tr>
<th>Main criteria related to the function</th>
<th>Rating</th>
<th>Indicators related to the main criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>R 6.3.2 Procedures to recognize exceptions are clearly defined.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S 6.3.3 Assessment reports are prepared and used as a reference for decision-making.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S 6.3.4 Written criteria exist for recognition of the reports and/or decisions of other NRAs (if applicable).</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S 6.3.5 Written guidelines for assessment of applications are available.</td>
</tr>
</tbody>
</table>

6.4 There is a requirement for changes to be submitted and assessed by the NRA prior to implementation.

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<thead>
<tr>
<th>Main criteria</th>
<th>Rating</th>
<th>Indicators related to the main criteria</th>
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</thead>
<tbody>
<tr>
<td>R 6.4.1 Changes are assessed.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S 6.4.2 Written guidelines for applicants are available that define the types and scopes of changes and documentation required.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S 6.4.3 Written guidelines for assessment are available based on the type of change.</td>
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</tr>
</tbody>
</table>

6.5 Appropriate assessment expertise exists.

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<thead>
<tr>
<th>Main criteria</th>
<th>Rating</th>
<th>Indicators related to the main criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>R 6.5.1 Access to experts (internal and/or external) for assessment of plasma-derived products (preclinical, clinical and quality data) is assured, and lists staff and/or experts with relevant qualifications and experience.</td>
<td></td>
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</tr>
</tbody>
</table>

6.6 Clear and comprehensive information on authorized plasma-derived products is available.

<table>
<thead>
<tr>
<th>Main criteria</th>
<th>Rating</th>
<th>Indicators related to the main criteria</th>
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</thead>
<tbody>
<tr>
<td>R 6.6.1 The product information made available is approved.</td>
<td></td>
<td></td>
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<tr>
<td>Main criteria related to the function</td>
<td>Rating*</td>
<td>Indicators related to the main criteria</td>
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<tr>
<td></td>
<td>Main criteria</td>
<td>Indicator</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>6.6.2 A summary of product characteristics (SPC) or equivalent information is available for all plasma-derived products.</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>6.6.3 SPC-like information is regularly updated and publicly available.</td>
</tr>
<tr>
<td>6.7 A list of authorized products exists.</td>
<td>R</td>
<td>R 6.7.1 A list of authorized products is made available where needed.</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>6.7.2 A list of authorized products is publicly available.</td>
</tr>
</tbody>
</table>

* R = required; S = suggested.

### 7. Regulatory oversight of associated substances and medical devices including in vitro diagnostics

**Applicable to associated substances and medical devices including in vitro diagnostics**

<table>
<thead>
<tr>
<th>Main criteria related to the function</th>
<th>Rating*</th>
<th>Indicators related to the main criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Main criteria</td>
<td>Indicator</td>
</tr>
<tr>
<td>7.1 Legal provisions exist for regulatory oversight of the relevant associated substances and medical devices.</td>
<td>R</td>
<td>R 7.1.1 Premarket review and approval is required for in vitro diagnostics and screening test kits used for donor selection, testing of blood and blood components for therapeutic use, and/or for further manufacturing of plasma-derived products (e.g. tests for donor haemoglobin, tests for infectious disease markers).</td>
</tr>
</tbody>
</table>
Table continued

<table>
<thead>
<tr>
<th>Main criteria related to the function</th>
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<th>Indicators related to the main criteria</th>
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<tbody>
<tr>
<td></td>
<td>Main criteria</td>
<td>Indicator</td>
</tr>
<tr>
<td></td>
<td>R 7.1.2</td>
<td>Premarket review and approval is required for medical devices involved in the manufacture of blood components (e.g. apheresis machines).</td>
</tr>
<tr>
<td></td>
<td>R 7.1.3</td>
<td>Premarket review and approval is required for associated substances (e.g. anticoagulants, additive solutions).</td>
</tr>
<tr>
<td></td>
<td>R 7.1.4</td>
<td>The NRA has the enforcement power to investigate and act against marketed products and involved companies that do not comply with the requirements.</td>
</tr>
<tr>
<td>7.2 Systems for premarket review and approval of associated substances and relevant medical devices are established and operational.</td>
<td>R 7.2.1</td>
<td>The capability exists to perform science-based risk assessments and risk management.</td>
</tr>
<tr>
<td></td>
<td>R 7.2.2</td>
<td>Premarket review includes an assessment of quality, safety and effectiveness.</td>
</tr>
<tr>
<td></td>
<td>R 7.2.3</td>
<td>Advice for applicants on content (data requirements), format and procedures for submitting an application exists.</td>
</tr>
<tr>
<td></td>
<td>R 7.2.4</td>
<td>If decentralized, roles and responsibilities of the bodies involved are defined and there is a mechanism for information exchange between the control authority and the NRA.</td>
</tr>
</tbody>
</table>
Table continued

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<thead>
<tr>
<th>Main criteria related to the function</th>
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<tbody>
<tr>
<td></td>
<td>Main criteria</td>
<td>Indicator</td>
</tr>
<tr>
<td>S 7.2.5 Written guidelines for product assessments exist.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.3 Appropriate assessment expertise is available.</td>
<td>R</td>
<td>R 7.3.1 Access to experts with relevant qualifications and experience (internal and/or external) for assessment of blood and blood components (preclinical, clinical and quality data) is established.</td>
</tr>
<tr>
<td>S 7.3.2 Written procedures for selection, management and use of external experts are in place.</td>
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</table>

* R = required; S = suggested.

8. Access to a laboratory independent of manufacturers

Applicable to blood, blood components, plasma-derived medicinal products, associated substances, and medical devices including in vitro diagnostics

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<thead>
<tr>
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<tbody>
<tr>
<td></td>
<td>Main criteria</td>
<td>Indicator</td>
</tr>
<tr>
<td>8.1 Access by the NRA to an NCL independent of the manufacturer(s) is established.</td>
<td>R</td>
<td>R 8.1.1 Policy and operational agreements are in place for use of any external control laboratories.</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>R 8.1.2 Adequate testing plans, testing procedures and related documentation are available.</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>R 8.1.3 Responsibilities for testing in the pre-licensing and post-licensure period are clearly defined.</td>
</tr>
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<td>Main criteria related to the function</td>
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<td>Indicators related to the main criteria</td>
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<td></td>
<td>Main criteria</td>
<td>Indicator</td>
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<tr>
<td>8.1.4 The NCL is involved in defining the specifications and analytical methods during assessment of marketing authorizations.</td>
<td>S</td>
<td>8.1.4</td>
</tr>
<tr>
<td>8.2 Appropriate organization and financial support from management ensure the implementation of adequate testing programmes (including documentation) using appropriate equipment, and qualified and experienced staff.</td>
<td>R</td>
<td>R 8.2.1 Written testing procedures and related documentation are in place.</td>
</tr>
<tr>
<td></td>
<td>R 8.2.2 A re-test policy is established.</td>
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<tr>
<td></td>
<td>R 8.2.3 A strategy for the introduction and validation of new or improved tests exists.</td>
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<td></td>
<td>R 8.2.4 Reporting and issuance to the NRA of all critical results including out-of-specifications handling is implemented.</td>
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<tr>
<td></td>
<td>S 8.2.5 Document control is established.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S 8.2.6 SOPs, test procedures, sample handling and data management are organized.</td>
<td></td>
</tr>
<tr>
<td>8.3 An externally accredited quality management system (QMS) is in place in the laboratory.</td>
<td>S</td>
<td>S 8.3.1 A quality policy and quality manual exist.</td>
</tr>
<tr>
<td></td>
<td>S 8.3.2 A qualified quality manager is designated and a QMS is in operation.</td>
<td></td>
</tr>
<tr>
<td>8.4 Equipment documentation is in place.</td>
<td>R</td>
<td>R 8.4.1 Calibration and maintenance schedules are available.</td>
</tr>
<tr>
<td></td>
<td>R 8.4.2 Validation protocols are available.</td>
<td></td>
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<tr>
<td></td>
<td>S 8.4.3 Equipment selection processes are documented and unique equipment identification (ID) is in place.</td>
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<td>Main criteria related to the function</td>
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<td>Indicators related to the main criteria</td>
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<td>Main criteria</td>
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<tr>
<td></td>
<td>S</td>
<td>8.4.4 Commissioning records (i.e.</td>
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<tr>
<td></td>
<td></td>
<td>installation and qualification)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>are available.</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>8.4.5 Operation manuals and logs exist.</td>
</tr>
<tr>
<td>8.5 Human resource management is</td>
<td>R</td>
<td>8.5.1 Qualified and experienced</td>
</tr>
<tr>
<td>implemented.</td>
<td></td>
<td>staff members with defined responsibilities and competencies are available.</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>8.5.2 A staff training plan is</td>
</tr>
<tr>
<td></td>
<td></td>
<td>developed and implemented.</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>8.5.3 The impact of staff training is</td>
</tr>
<tr>
<td></td>
<td></td>
<td>monitored.</td>
</tr>
<tr>
<td>8.6 An audit and review system exists.</td>
<td>S</td>
<td>8.6.1 Comprehensive internal audit</td>
</tr>
<tr>
<td></td>
<td></td>
<td>and review systems are in place.</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>8.6.2 Documentation of actions taken as a result of audits is available.</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>8.6.3 The laboratory is audited by</td>
</tr>
<tr>
<td></td>
<td></td>
<td>external organizations.</td>
</tr>
<tr>
<td>8.7 A validation policy for the</td>
<td>R</td>
<td>8.7.1 A validation programme for non-compendial tests is available.</td>
</tr>
<tr>
<td>introduction of tests is implemented.</td>
<td></td>
<td>8.7.2 Procedures exist for transfers of validated methods (i.e. between the manufacturer and the regulator).</td>
</tr>
<tr>
<td>8.8 A general safety programme exists.</td>
<td>R</td>
<td>8.8.1 Lists of hazardous substances are available.</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>8.8.2 Responsible staff members are designated.</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>8.8.3 A full safety programme exists.</td>
</tr>
</tbody>
</table>
### Table continued

<table>
<thead>
<tr>
<th>Main criteria related to the function</th>
<th>Rating*</th>
<th>Indicators related to the main criteria</th>
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<tbody>
<tr>
<td></td>
<td>Main criteria</td>
<td>Indicator</td>
</tr>
<tr>
<td>8.9 A policy for use of reference standards and reagents exists.</td>
<td>R</td>
<td>R</td>
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<td></td>
<td>R</td>
<td></td>
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<td></td>
<td>R</td>
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<tr>
<td></td>
<td>S</td>
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</tr>
<tr>
<td>8.10 Data trends are monitored and analysed.</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>8.11 Participation in international proficiency schemes and collaborative studies is organized.</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>8.12 Regulatory outcome of testing is analysed and used as a basis for decision-making.</td>
<td>R</td>
<td>R</td>
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<td>R</td>
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<td></td>
<td>R</td>
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* R = required; S = suggested.
9. Control of clinical trials

Applicable to blood, blood components, plasma-derived medicinal products, associated substances, and medical devices including in vitro diagnostics

<table>
<thead>
<tr>
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<tbody>
<tr>
<td></td>
<td>Main criteria</td>
<td>Indicator</td>
</tr>
<tr>
<td>9.1 Applicable legal provision for the regulation of biomedical research in human subjects exists.</td>
<td>R</td>
<td>R 9.1.1 An authorization system for clinical trials is required.</td>
</tr>
<tr>
<td></td>
<td>R 9.1.2 The scope and requirements for regulation of clinical trials are defined.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R 9.1.3 The NRA has the enforcement power for the authorization, suspension and withdrawal of clinical trials.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R 9.1.4 Legal provisions are in place to assure an ethical oversight of clinical trials.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R 9.1.5 Compliance with principles of good clinical practice (GCP) is mandatory.</td>
<td></td>
</tr>
<tr>
<td>9.2 A system for authorization of clinical trials is operational.</td>
<td>R</td>
<td>R 9.2.1 A system is established for clinical trial assessment and authorization.</td>
</tr>
<tr>
<td></td>
<td>R 9.2.2 An inspection system is established to verify compliance with the principles of GCP.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R 9.2.3 Expertise is available from within or outside the NRA.</td>
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</tr>
<tr>
<td></td>
<td>S 9.2.4 Written guidelines for assessment of clinical trials and changes are implemented.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S 9.2.5 Written guidelines and forms on the data requirements, the format and procedures for submitting a clinical trial application are available to sponsors.</td>
<td></td>
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<tr>
<td>Main criteria related to the function</td>
<td>Rating*</td>
<td>Indicators related to the main criteria</td>
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<tr>
<td></td>
<td>Main criteria</td>
<td>Indicator</td>
</tr>
<tr>
<td>S 9.2.6 Provision for scientific advice (e.g. preclinical and clinical) on the design of clinical trials or issues related to the submission of appropriate data is in place.</td>
<td>S 9.2.7 There are written guidelines for GCP.</td>
<td></td>
</tr>
<tr>
<td>9.3 Data requirements for clinical trial applications are defined.</td>
<td>R</td>
<td>R 9.3.1 Production and quality control of the clinical candidate material (e.g. product characterization, laboratory specimens) are included.</td>
</tr>
<tr>
<td></td>
<td>R 9.3.2 Provision for preclinical data exists.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R 9.3.3 Assessment of the clinical trial protocol with respect to patient safety and informed consent is performed.</td>
<td></td>
</tr>
<tr>
<td>9.4 Assurance of ethical oversight exists.</td>
<td>R</td>
<td>R 9.4.1 A system of independent ethical review and approval exists in accordance with the principles of GCP.</td>
</tr>
<tr>
<td></td>
<td>S 9.4.2 Ethics committees (e.g. an Institutional Review Board) are formally defined, including their composition.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S 9.4.3 The ethics committees include members external to the concerned institution.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S 9.4.4 The roles and duties of ethics committees to oversee clinical trials are outlined.</td>
<td></td>
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</tbody>
</table>

* R = required; S = suggested.
## 10. System for lot release of plasma-derived medicinal products

*Applicable to plasma-derived medicinal products and donor screening tests*

<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td></td>
<td>Main criteria</td>
<td>Indicator</td>
</tr>
<tr>
<td>10.1 Legal provisions for official lot release certification are in place.</td>
<td>R</td>
<td>10.1.1 The NRA has the authority to issue lot release certificates and the enforcement power to suspend or revoke lot release.</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>10.1.2 The NRA has the legal authority to perform lot release and/or have in place a policy and criteria for acceptance of lot release performed by another NRA (e.g. a lot release certificate from the country of origin).</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>10.1.3 Written criteria for exemption from lot release exist.</td>
</tr>
<tr>
<td>10.2 A lot release system is established and operational.</td>
<td>R</td>
<td>10.2.1 Lot release protocols and procedures are established and/or acceptance of lot release performed by another NRA is in place.</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>10.2.2 Lot release is based at a minimum on review of summary lot-specific data.</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>10.2.3 Qualified staff members (i.e. staff with relevant qualifications, training and experience) are available to perform lot release.</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>10.2.4 Testing policy and test protocols including acceptance criteria are defined.</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>10.2.5 Records on lot release are maintained.</td>
</tr>
</tbody>
</table>
### Table continued

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<tr>
<th>Main criteria related to the function</th>
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<tbody>
<tr>
<td></td>
<td>Main criteria</td>
<td>Indicator</td>
</tr>
<tr>
<td>R 10.2.6 Procedures for communication with the product manufacturer are defined.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R 10.2.7 Written procedures and guidelines (including templates of certificates), checklists, and/or SOPs are developed and used to review summary lot protocols and are implemented for the lot release process.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S 10.2.8 Testing procedures are externally accredited.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.3 A quality management system for official lot release is implemented.</td>
<td>R</td>
<td>R 10.3.1 The laboratory that performs lot release within or for the NRA complies with core function 8.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S 10.3.2 Appropriate data collection and analysis (e.g. lot-to-lot consistency, trend analysis) is implemented.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S 10.3.3 Continual review and scientific dialogue exist with the manufacturers and product review experts on issues of quality test results.</td>
</tr>
<tr>
<td>10.4 Access to product-related documentation to guide particular areas of scrutiny in lot release is possible.</td>
<td>R</td>
<td>R 10.4.1 Approved relevant marketing authorization and updates are available.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R 10.4.2 Access to complaints and adverse event (AE) reports is possible.</td>
</tr>
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<td></td>
<td><strong>Main criteria</strong></td>
<td><strong>Indicator</strong></td>
</tr>
<tr>
<td></td>
<td><strong>R</strong></td>
<td>10.4.3 Access to the manufacturer's batch records is possible.</td>
</tr>
<tr>
<td></td>
<td><strong>R</strong></td>
<td>10.4.4 Access to inspection reports is possible.</td>
</tr>
</tbody>
</table>

*R = required; S = suggested.

### 11. Regulatory inspections and enforcement activities

*Applicable to blood, blood components, plasma-derived medicinal products, associated substances, and medical devices including in vitro diagnostics*

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<tr>
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<tr>
<td></td>
<td><strong>Main criteria</strong></td>
<td><strong>Indicator</strong></td>
</tr>
<tr>
<td>11.1 Legal provision exists to inspect premises where regulated activities are performed in order to assess and enforce compliance with the applicable laws, regulations and standards.</td>
<td><strong>R</strong></td>
<td>11.1.1 A mandate exists for inspections by the NRA and enforcement of compliance with principles of GMP, GDP and other standards.</td>
</tr>
<tr>
<td></td>
<td><strong>R</strong></td>
<td>11.1.2 Applicable standards and practices are defined in legal provisions.</td>
</tr>
<tr>
<td></td>
<td><strong>R</strong></td>
<td>11.1.3 The NRA has the authority to take enforcement action against the accountable companies or persons that are not in compliance.</td>
</tr>
<tr>
<td></td>
<td><strong>R</strong></td>
<td>11.1.4 The NRA has the authority to sample products, manufacturing materials and records if necessary.</td>
</tr>
<tr>
<td></td>
<td><strong>R</strong></td>
<td>11.1.5 The NRA has the authority to recall products.</td>
</tr>
<tr>
<td></td>
<td><strong>R</strong></td>
<td>11.1.6 Provisions exist for conflict of interest and confidentiality.</td>
</tr>
<tr>
<td>Main criteria related to the function</td>
<td>Rating</td>
<td>Indicators related to the main criteria</td>
</tr>
<tr>
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</tr>
<tr>
<td></td>
<td>Main</td>
<td>Indicator</td>
</tr>
<tr>
<td>11.2 Inspection and enforcement systems are established and operational.</td>
<td>R</td>
<td>R 11.2.1 Established policies and programmes exist for conducting inspections of all regulated activities.</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>R 11.2.2 An inspection plan exists with adequate human and financial resources for conducting inspections at appropriate intervals.</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>R 11.2.3 The NRA maintains files of each inspection, including the inspection report and final decisions taken.</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>R 11.2.4 There is an established process for appropriate regulatory action to address inspectional findings (e.g. recall of products, amended licences).</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>R 11.2.5 If the mechanism is adopted, provisions exist for acceptance of external inspectorates according to internationally recognized standards.</td>
</tr>
<tr>
<td>11.3 Inspectors with appropriate expertise and qualifications are available.</td>
<td>R</td>
<td>R 11.3.1 Inspectors have the appropriate expertise and training to conduct inspections of blood establishments, and of manufacturers and distributors of plasma-derived products.</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>R 11.3.2 Training of inspectors includes specific aspects related to the activities of relevant establishments.</td>
</tr>
<tr>
<td>Main criteria related to the function</td>
<td>Rating*</td>
<td>Indicators related to the main criteria</td>
</tr>
<tr>
<td>--------------------------------------</td>
<td>---------</td>
<td>---------------------------------------</td>
</tr>
<tr>
<td>S 11.3.3 Use of a team approach is possible in order to include specialized knowledge and expertise in specific products where needed.</td>
<td>R 11.4.1 Written procedures exist for conducting inspections (inspection manual) and following up on deficiencies and/or violations.</td>
<td></td>
</tr>
<tr>
<td>S 11.4.2 An established procedure (e.g. periodic internal and external audits) exists to monitor the inspection process.</td>
<td>R 11.4.3 Monitoring of timelines and indicated actions is implemented.</td>
<td></td>
</tr>
<tr>
<td>R 11.5.1 Policy and procedures for a recall system including product disposition exist.</td>
<td>R 11.5.2 The recall system is based on defined action and documented communication to the appropriate level of the distribution system.</td>
<td></td>
</tr>
<tr>
<td>R 11.5.3 A feedback mechanism exists to confirm that appropriate action (including destruction when necessary) has been taken at all appropriate levels.</td>
<td>R 11.5.4 Full lot traceability is in place.</td>
<td></td>
</tr>
</tbody>
</table>

*R = required; S = suggested.
### 12. Vigilance systems

*Applicable to blood, blood components, plasma-derived medicinal products, associated substances, and medical devices including in vitro diagnostics*

<table>
<thead>
<tr>
<th>Main criteria related to the function</th>
<th>Rating</th>
<th>Main criteria</th>
<th>Indicator</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>12.1 Legal provisions for a national vigilance system exist.</strong></td>
<td>R</td>
<td>R 12.1.1 The NRA has a legal mandate and enforcement power for mandatory reporting elements of the national vigilance system.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>12.1.2 The NRA has the authority to specify reporting of adverse events (AEs) and adverse reactions (ARs) within the national vigilance system.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>12.1.3 Authority exists to require the marketing authorization holder to perform a specific study of safety and/or effectiveness in the post-marketing period.</td>
<td></td>
</tr>
<tr>
<td><strong>12.2 National vigilance systems for the monitoring and management of AE and AR are established and operational.</strong></td>
<td>R</td>
<td>R 12.2.1 Roles and responsibilities of the key parties, the NRA, and surveillance staff involved in AE and AR monitoring and management activities are clearly defined and documented.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>12.2.2 Guidelines exist and are published and accessible (i.e. distributed or available when needed) to all staff involved in AE and AR surveillance.</td>
<td></td>
</tr>
</tbody>
</table>
Table continued

<table>
<thead>
<tr>
<th>Main criteria related to the function</th>
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<tbody>
<tr>
<td></td>
<td>Main indicator</td>
<td></td>
</tr>
<tr>
<td>S 12.2.3</td>
<td>Guidelines include the following:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>a. objectives of the system;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>b. a list of AEs and ARs to be reported;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>c. case definitions for all AEs and ARs to be reported;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>d. information on how to report AEs and ARs for all blood, blood components, plasma-derived products, associated substances, and medical devices including in vitro diagnostics (i.e. who should report; how, where and when reports should be sent);</td>
<td></td>
</tr>
<tr>
<td></td>
<td>e. the process for analysing data and providing feedback to relevant staff and key parties;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>f. the process for investigating and responding to serious AEs and ARs (including who should be in charge of the investigation);</td>
<td></td>
</tr>
<tr>
<td></td>
<td>g. the process for informing patients, parents, the community and country (where relevant) of the findings of an investigation and relevant actions.</td>
<td></td>
</tr>
<tr>
<td>S 12.2.4</td>
<td>A standardized reporting form exists with comprehensive information to monitor AEs and ARs.</td>
<td></td>
</tr>
</tbody>
</table>
### Table continued

<table>
<thead>
<tr>
<th>Main criteria related to the function</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Main criteria</td>
<td>Indicator</td>
</tr>
<tr>
<td>S 12.2.5 A system is established for providing periodic feedback on AEs and ARs, including summary and specific investigation reports from the national to all levels (including health-facility level).</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>12.3 Guidance on AE and AR monitoring and management is provided to appropriate staff.</td>
<td>S</td>
<td>S 12.3.1 Guidelines and templates on AE and AR reporting and monitoring are provided to appropriate staff dealing with AE and AR.</td>
</tr>
<tr>
<td>12.4 There is demonstrated capacity to detect, investigate and take action regarding significant AEs and ARs.</td>
<td>R</td>
<td>R 12.4.1 The NRA is regularly informed of data relevant to the quality and safety of blood products including: a. blood transfusion safety; b. transmissible disease surveillance data; c. device failures.</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>12.4.2 Manufacturers are required to inform the NRA of any new safety issues or marketing and/or regulatory decisions taken in other countries.</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>12.4.3 Procedures for initiating corrective and/or regulatory action (e.g. recall) are available.</td>
</tr>
</tbody>
</table>
### Table continued

<table>
<thead>
<tr>
<th>Main criteria related to the function</th>
<th>Rating*</th>
<th>Indicators related to the main criteria</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>R 12.4.4 There is documented capacity to investigate AEs and ARs, for example:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>a. routine reporting of AEs and ARs according to established guidelines and/or SOPs;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b. a clear understanding and adequate training among key parties of respective roles and responsibilities;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c. access to resources (personnel, laboratory) to conduct comprehensive investigations.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R 12.4.5 Case investigations are timely and complete, for example:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>a. timelines are established for prompt investigation and preliminary reporting related to serious adverse reactions;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b. investigation is thorough and findings are clearly described.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S 12.4.6 There is a demonstrated reporting system (active or passive, sentinel or universal) with satisfactory sensitivity, for example:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>a. annual number of reports;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b. reporting rate;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>c. breakdown of reports by types of AE, age group, districts etc.</td>
</tr>
</tbody>
</table>

* R = required; S = suggested.
<table>
<thead>
<tr>
<th>Main criteria related to the function</th>
<th>Rating*</th>
<th>Indicators related to the main criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13.1 The NRA ensures that standards for traceability and record-keeping are in place for all aspects of manufacturing and distribution.</td>
<td>R R 13.1.1</td>
<td>A requirement exists for manufacturers to implement methods and maintain records that enable traceability, including: a. for manufacturers of blood products, traceability from donor to recipient and vice versa; b. ensuring the integrity of manufacturing records and completeness of distribution records. R 13.1.2</td>
</tr>
</tbody>
</table>

*R = required; S = suggested.

<table>
<thead>
<tr>
<th>Main criteria related to the function</th>
<th>Rating*</th>
<th>Indicators related to the main criteria</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14.1 A national policy to facilitate international cooperation and harmonization is implemented.</td>
<td>S S 14.1.1</td>
<td>A national policy and/or strategy on international interactions exist, e.g. information sharing on product approvals, safety data and policy initiatives.</td>
</tr>
<tr>
<td>Main criteria related to the function</td>
<td>Rating*</td>
<td>Indicators related to the main criteria</td>
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<td>----------------------------------------</td>
</tr>
<tr>
<td></td>
<td>Main criteria</td>
<td>Indicator</td>
</tr>
<tr>
<td>S 14.1.2 Agreements exist between the NRA and other international organizations and regulatory authorities.</td>
<td>S 14.1.3 The NRA participates in international harmonization initiatives and forums.</td>
<td></td>
</tr>
<tr>
<td>14.2 Sharing of risk information with international organizations and other regulatory authorities is implemented.</td>
<td>R R 14.2.1 Ability is shown by the NRA to participate in international risk management efforts when needed.</td>
<td>S 14.2.2 The NRA has the ability to engage in international risk assessment when needed, e.g. access to epidemiological data, expertise in risk assessment.</td>
</tr>
<tr>
<td></td>
<td>S 14.2.3 The capacity or expertise to access epidemiological data and formally assess risks is available.</td>
<td>S 14.2.4 Documented procedures for the timely sharing of risk information internationally exist.</td>
</tr>
<tr>
<td></td>
<td>S 14.2.5 Records are kept of risk information that has been exchanged.</td>
<td></td>
</tr>
</tbody>
</table>

* R = required; S = suggested.
Authors and acknowledgements

The drafting group was formed of Members of the WHO Blood Regulators Network (BRN) and the WHO Blood Products and Related Biologicals programme, Quality Assurance and Safety: Medicines, World Health Organization:

Dr F. Agbanyo, Health Canada, Canada; Dr J. Epstein, United States Food and Drug Administration, USA; Dr P. Ganz, Health Canada, Canada; Dr M. Heiden, Paul-Ehrlich-Institute, Germany; Dr M. Jutzi, Swissmedic, Switzerland; Dr G. Michaud, United States Food and Drug Administration, USA; Dr A. Padilla, World Health Organization, Geneva, Switzerland; Dr I. Prosser, Therapeutic Goods Administration, Australia; Dr I. Sainte-Marie, French Agency for the Safety of Health Products (AFSSAPS), France; Dr C. Schärer, Swissmedic, Switzerland; Professor R. Seitz, Paul-Ehrlich-Institute, Germany; Dr G. Smith, Therapeutic Goods Administration, Australia; Dr P. Zorzi, AFSSAPS, France.

Existing WHO evaluation templates for vaccines and medicinal products were consulted in developing this tool. The first consolidated draft was discussed at the Blood and Blood Products Workshop of the 14th International Conference of Drug Regulatory Authorities, Singapore, 2010, where it was supported for consideration by WHO Member States. More than 90 national regulatory agencies were represented at the Conference.

Through a global consultation process involving all WHO regions, regulators were encouraged to contribute their self-assessments and comments on the usefulness of the tool to help towards its finalization. Thanks are due to the WHO regional offices for their support in this process.

Valuable inputs in the form of comments and self-assessment feedback were received from the following national agencies (in alphabetical order by country):

Blood Bank Directorate, Ministry of Public Health, Afghanistan; Administración Nacional de Medicamentos, Alimentos y Tecnología Médica (ANMAT), Argentina; Scientific Center of Drug and Medical Technologies Expertise (SCDMTE), Armenia; European Commission, Directorate General for Health and Consumer Affairs (SANCO), Belgium; Gerência Geral de Sangue, outros Tecidos, Células e Órgãos, Agência Nacional de Vigilância Sanitária (ANVISA), Brazil; Department of Drug Registration, State Food and Drug Administration, China; Instituto Nacional de Salud (INS) and Instituto Nacional de Vigilancia de Medicamentos y Alimentos (INVIMA), Ministerio de la Protección Social, Colombia; Centro para el Control Estatal de la Calidad de los Medicamentos (CECMED), Cuba; Danish Medicines Agency, Denmark; The Minister’s Technical Office, Ministry of Health, Egypt; Laboratory Services Department, Food and Drugs Board, Ghana; Central Drugs Standard Control Organization, Ministry of Health and Family Welfare, India; National Agency of Drug and Food Control
(NADFC), Indonesia; Food and Drug Organization, Islamic Republic of Iran; Division of Blood and Blood Products, Ministry of Health, Labour and Welfare, Japan; National Blood Service, Ministry of Health, Latvia; Comisión Federal para la Protección contra Riesgos Sanitarios (COFEPRIS), Mexico; Department of Health and Social Affairs, Micronesia (Federated States of); Department of Drug Administration (DDA), Ministry of Health and Population, Nepal; Medicines Evaluation Board, the Netherlands; Centro Nacional de Diagnóstico y Referencia (CNDR), Ministerio de Salud de Nicaragua (MINSA), Nicaragua; National Agency for Food and Drug Administration and Control (NAFDAC), Nigeria; Programa Nacional de Sangre, Ministerio de Salud Pública y Bienestar Social, Paraguay; Centre for Product Registration, National Pharmaceutical Control Bureau, Ministry of Health, Malaysia; Korea Food and Drug Administration (KFDA), Republic of Korea; Saudi Food and Drug Authority, Saudi Arabia; Direction de la Pharmacie et des Laboratoires, Ministère de la Santé, Senegal; Medicines and Medical Devices Agency, Serbia; Department of Health, South Africa; National Drug Quality Control Laboratory, Medicines and Poisons Board, Sudan; Medical Products Agency, Sweden; Ministry of Health, Syrian Arab Republic; Institute of Biological Products, Department of Medical Sciences, Ministry of Public Health, Thailand; Sharjah Blood Transfusion and Research Centre, Ministry of Health, United Arab Emirates.

All comments received were reviewed by the drafting group and a final proposed version was submitted to the sixty-second Expert Committee on Biological Standardization. Special thanks are due to the Members of the Committee, who provided valuable advice and agreed to the adoption of this document:

Dr. J. Epstein, Center for Biologics Evaluation and Research, Food and Drug Administration, USA; Dr. E. Griffiths, Biologics and Genetic Therapies Directorate, Health Canada, Canada; Mrs. T. Jivapaisarnpong, Institute of Biological Products, Department of Medical Sciences, Ministry of Public Health, Thailand; Dr. H. Klein, National Institutes of Health, USA; Dr. P. Minor, National Institute for Biological Standards and Control, England; Dr. F.M. Moftah, National Blood Transfusion Service, Ministry of Health, Egypt; Dr. J. Petricciani, International Association for Biologicals, USA; Dr. L.S. Slamet, National Agency of Drug and Food Control (NADFC), Indonesia; Dr. P. Strengers, Sanquin Foundation, the Netherlands; Professor H. Yin, Center for Drug Evaluation, State Food and Drug Administration, China.

For further information contact:
Department of Essential Medicines and Health Products
World Health Organization
Bibliography


Annex 8

Biological substances: WHO International Standards and Reference Reagents

A list of WHO International Standards and Reference Reagents for biological substances was issued in WHO Technical Report Series, No. 897, 2000 (Annex 4) with an updated version available at: http://www.who.int/biologicals. Copies of the list may be obtained from appointed sales agents for WHO publications or from WHO Press, World Health Organization, 1211 Geneva 27, Switzerland (tel.: +41 22 791 3264; fax: +41 22 791 4857; e-mail: bookorders@who.int; web site: http://www.who.int/bookorders).

At its meeting in October 2011, the Expert Committee made the changes shown below to the previous list.

Vaccines and related substances; blood products and related substances; biotherapeutics other than blood products; and in vitro diagnostic device reagents are held and distributed by the International Laboratory for Biological Standards, NIBSC, Potters Bar, Herts, England. Antibiotic Reference Preparations are held by the European Department for the Quality of Medicines, Council of Europe, 7 allée Kastner, CS 30026 F-67081, Strasbourg, France.

Additions

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Activity</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antibiotics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dihydrostreptomycin</td>
<td>19 425 IU per vial</td>
<td>Third WHO International Standard</td>
</tr>
<tr>
<td><strong>Biotherapeutics other than blood products</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transforming growth factor beta-3</td>
<td>19 000 IU per ampoule</td>
<td>First WHO International Standard</td>
</tr>
<tr>
<td><strong>Blood products and related substances</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-human neutrophil antigen-1a antibody</td>
<td>No assigned value; however, a 1-in-4 dilution should define the minimum potency specification for anti-HNA-1a detection</td>
<td>First WHO Reference Reagent</td>
</tr>
<tr>
<td>Preparation</td>
<td>Activity</td>
<td>Status</td>
</tr>
<tr>
<td>--------------------------------------</td>
<td>-----------------------------------------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>Blood group genotyping</td>
<td>No assigned values</td>
<td>First WHO Reference Reagents</td>
</tr>
<tr>
<td>Factor VIII/von Willebrand factor (VWF), plasma</td>
<td>1.03 IU per ampoule for VWF propeptide</td>
<td>Sixth WHO International Standard</td>
</tr>
<tr>
<td>Fibrinogen, plasma</td>
<td>2.7 mg per ampoule</td>
<td>Third WHO International Standard</td>
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</tbody>
</table>

**In vitro diagnostic device reagents**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Activity</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-<em>Trypanosoma cruzi</em> group I antibodies, human</td>
<td>1 IU per ml</td>
<td>First WHO International Standard</td>
</tr>
<tr>
<td>Anti-<em>Trypanosoma cruzi</em> group II antibodies, human</td>
<td>1 IU per ml</td>
<td>First WHO International Standard</td>
</tr>
<tr>
<td>Epstein–Barr virus, for NAT-based assays</td>
<td>5 000 000 IU per ml</td>
<td>First WHO International Standard</td>
</tr>
<tr>
<td>Hepatitis B virus DNA, for NAT-based assays</td>
<td>850 000 IU per ml</td>
<td>Third WHO International Standard</td>
</tr>
<tr>
<td>Hepatitis B virus genotypes, for HBsAg assays</td>
<td>No assigned values</td>
<td>First WHO International Reference Panel</td>
</tr>
<tr>
<td>Hepatitis C virus RNA, for NAT-based assays</td>
<td>260 000 IU per ml</td>
<td>Fourth WHO International Standard</td>
</tr>
<tr>
<td>Hepatitis E virus RNA, for NAT-based assays</td>
<td>250 000 IU per ml</td>
<td>First WHO International Standard</td>
</tr>
<tr>
<td>HIV-1 RNA, for NAT-based assays</td>
<td>185 000 IU per ml</td>
<td>Third WHO International Standard</td>
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**Vaccines and related substances**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Activity</th>
<th>Status</th>
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</thead>
<tbody>
<tr>
<td>Anti-pneumococcal antibodies, human, serum</td>
<td>serotype 1, 8.5 µg/ml serotype 3, 1.45 µg/ml serotype 4, 3.33 µg/ml serotype 5, 7.51 µg/ml serotype 6A, 3.93 µg/ml serotype 6B, 9.05 µg/ml serotype 7F, 8.3 µg/ml serotype 9V, 6.44 µg/ml serotype 14, 37.99 µg/ml serotype 18C, 7.3 µg/ml serotype 19A, 13.87 µg/ml serotype 19F, 14.61 µg/ml serotype 23F, 5.95 µg/ml</td>
<td>First WHO International Standard</td>
</tr>
<tr>
<td>Meningocococal serogroup C polysaccharide</td>
<td>1.192 mg per ampoule</td>
<td>First WHO International Standard</td>
</tr>
</tbody>
</table>