WHO Technical Report Series
932

WHO EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION

Fifty-fifth report

This report presents the recommendations of a WHO Expert Committee commissioned to coordinate activities leading to the adoption of international recommendations for the production and control of vaccines and other biologicals and the establishment of international biological reference materials. The report starts with a discussion of general issues brought to the attention of the Committee and provides information on the status and development of reference materials for various antibodies, antigens, blood products and related substances, cytokines, growth factors, and endocrinological substances. The second part of the report, of particular relevance to manufacturers and national regulatory authorities, contains guidelines on the production and quality control of candidate tetravalent dengue virus vaccines and recommendations for the preparation, characterization and establishment of international and other biological reference standards. Also included are a list of recommendations, guidelines and other documents for biological substances used in medicine, and of international standards and reference reagent for biological substances.

ISBN 92 4 120932 1
9 789241 209328

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World Health Organization

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WHO Technical Report Series
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WHO Library Cataloguing-in-Publication Data

WHO Expert Committee on Biological Standardization (2004 : Geneva, Switzerland)
WHO Expert Committee on Biological Standardization : fifty-fifth report.

(WHO technical report series ; 932)

1.Biological products - standards 2.Immunologic factors - standards 3.Blood
4.Reagent kits, Diagnostic - standards 5.Reference standards 5.Guidelines I.Title
II.Series

ISBN 92 4 120932 1 (LC/NLM classification: QW 800)
ISSN 0512-3054

Typeset in China, Hong Kong Special Administrative Region
Printed in Singapore

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Geneva, 15–18 November 2004

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Introduction

The WHO Expert Committee on Biological Standardization met in Geneva from 15 to 18 November 2004. The meeting was opened on behalf of the Director-General by Mrs J. Phumaphi, Assistant Director-General.

Mrs Phumaphi emphasized the importance of the work of the Committee in preparing recommendations to assure safe and efficacious biological products and in establishing standard preparations important for global health. Fully functional national regulatory authorities that refer to WHO recommendations, norms and standards are essential to protect patients and improve global public health. She recalled that the Expert Committee on Biological Standardization, which had started its work as early as 1947, was one of the longest-standing WHO Committees. The Committee has been faced with the challenge of technological advancements while maintaining the highest possible standards for the quality, safety and efficacy of vaccines, biological therapeutics, blood products and selected in vitro diagnostic devices. The wide range of WHO biological reference standards improves the comparability of data in diverse fields of medical practice. Mrs Phumaphi encouraged the members to actively engage in the biological standardization work needed in their respective countries. The expertise and the experience of the participants represent an important resource that would be crucial to countries, for example in ensuring their preparedness to react to the threat of avian flu where expert guidance would facilitate the rapid availability of new vaccines as needed. She reminded the Committee of the immense contribution it had made recently, such as in the case of diagnosis of hepatitis B, which is still a threatening disease in parts of the world; some 2 billion people have been infected globally and, of these, about 360 million are believed to be chronically infected. The availability of new international reference materials for hepatitis B is a critical step in the development of appropriate standards needed for countries that lack direct access to ready-to-use reagents to evaluate diagnostics. Mrs Phumaphi emphasized the importance of promoting good manufacturing practices (GMP) for blood and blood products and pointed out that the implementation of GMP represents a very effective tool for assuring the safety and quality of all biological products.

Mrs Phumaphi underlined the continued rapid developments in the field of biologicals represented by a proposal at this meeting to establish the first international standard for a human genetic test. The application of molecular genomics to medical problems presents great opportunities but also considerable challenges.

Mrs Phumaphi reminded the members of the Committee that they did not represent organizations or governments, but had been invited by WHO because of their individual expertise. She invited all the participants to contribute actively in their respective capacities. Finally she reminded the Committee that all decisions made should be based on sound scientific principles.

General

Developments in biological standardization: WHO programmatic issues

The Committee was reminded that the context of its work is based on mandates by Member States to develop, establish and promote international standards for biological products. Within WHO two units are working together, namely, Quality Assurance and Safety of Biologicals (QSB), in the Department of Immunization, Vaccines and Biologicals, and Quality Assurance and Safety of Plasma Derivatives and Related Substances (QSD), in the Department of Essential Health Technologies. The QSB unit is primarily responsible for vaccines and biological therapeutics, whereas QSD is primarily responsible for blood products and related in vitro diagnostic devices. Since this integrated approach had been established 5 years earlier (in 1999), a review to determine whether this organizational structure was the optimal configuration for the future work of the Organization in the area of biological standardization was considered timely. The outcome of this internal review and the conclusions will be communicated to the Committee.

The impact of achievements in biological standardization was highlighted. It was noted that these achievements are not always immediately visible or easy to communicate to decision-makers outside the field and that one pressing challenge was to improve communication about why biological standardization matters. Recent examples that illustrate the impact of the work of the Committee included concerns from the public about the quality of oral poliomyelitis vaccine in Nigeria, the need for global regulatory harmonization to assist preparedness for vaccines against avian influenza, the new International Standard for hepatitis B surface antigen that defines the unitage for regulatory evaluations of diagnostic test kits around the world, and the precautionary policies specified in WHO guidance for managing the risks of transmissible spongiform encephalopathy (TSE) from biological and pharmaceutical products. The Committee agreed there was a need to develop advocacy materials targeted at

decision-makers to ensure that the case for biological standardization is better understood.

The Committee was informed of future WHO priorities, namely to put more emphasis on regional and country-based activities. This would be implemented through alterations to the structure of the 2006–2007 budget for the Organization, resulting in more resources and responsibilities for the regions and countries. The challenge in this changing environment is to strengthen the core normative activities in biological standardization.

Steps have been taken by the Secretariat to improve interactions with the key laboratories that provide essential support to the WHO biological standardization programme. Specifically a meeting was held in May 2004 with the WHO International Laboratories and WHO Collaborating Centres that conduct standardization studies for WHO. The outcome helped to align the priorities of WHO and the laboratories.

Crucial aspects for future WHO activities in biological standardization were identified. These included:

— the need to strengthen regional networks, training and funding in developing countries for biological standardization and quality control;
— to facilitate an efficient process whereby science continues to be integrated into regulations;
— to continue to improve collaboration with other international standard setting bodies; and
— to strengthen resources available for the area of work in blood products and related in vitro diagnostic devices.

A need was also identified to expand the skill base of the group responsible for biological standardization, namely the Expert Advisory Panel on Biological Standardization. The aim was to achieve the broadest international representations and diversity in knowledge, experience and approaches, and to improve the gender balance and the geographical representation.

Priorities were being addressed for establishing written standards to maintain the balance between development of new guidance and the revision of existing guidance depending upon WHO programmes and needs, and in the area of reference preparations to align international needs to the available production capacity of the international laboratories. Inputs into the prioritization process included the recommendations of this Committee, the International Conference of Drug Regulatory Authorities, the Strategic Advisory Group of Experts of

the Department of Immunization, Vaccines and Biologicals, the WHO regional advisers, and various specialized working groups established by the Secretariat.

Management and dissemination of all the accumulated knowledge is crucial. Because the output in terms of guidance documents and International Standards has to be made available as quickly as possible to interested parties, WHO is giving high priority to the publication process. The report of the last two meetings of the ECBS have been published more quickly in the WHO Technical Report Series than in the past, and the targeting of each technical report to national regulatory authorities was being improved. Summary reports of ECBS meetings have also been published in the journals *WHO Drug Information* and *Regulatory Affairs Journal Pharma*, and reports of WHO consultations have been published in journals such as *Vaccines* and *Biologicals*. This information was also posted on the WHO web site (see www.who.int/biologicals). The Biologicals web site was being modified to meet a new standard that incorporated WHO's corporate identity, aimed to improve navigation in general and allows access via links from the web pages of other units within the WHO web site.

The Committee was reminded that biological standardization remained a core activity for global health. The main focus for WHO in the near future would be improved support to regions and countries, the need for additional human resources, improved advocacy in general, expanded skills for oversight, improved alignment of priorities and the improvement of knowledge management.

The Committee welcomed the plans, but reiterated its concerns about the underfunded and understaffed units, especially that for blood and blood products, which may lead to difficulties in meeting expectations if there are no improvements in the near future.

Developments in biological standardization: vaccines and other biologicals

The Committee was informed of recent developments and action plans of the Secretariat for the coming years.

Acceptability of cell substrates

The range of cell substrates that could be used for the production of biological products, including vaccines, is broad. However the range of cell substrates widely accepted by national regulatory authorities, especially for vaccine production, is more limited. Historically there is a trend in preference from primary cells to diploid cells to non-tumorigenic continuous cells, as reflected in WHO recommendations

(WHO Technical Report Series, No. 878, 1998). A number of vaccine candidates under development are being produced on cell substrates that are not widely accepted by national regulatory authorities which raises a number of issues including the potential risk, if any, from residual cellular DNA. Currently limits are set for certain cell types, such as non-tumorigenic continuous cells, but a key issue is whether this risk management strategy is sufficient to ensure the safety of the new cell lines under consideration. At a conference organized by the International Association of Biologics and the US National Institute of Allergy and Infectious Diseases, held in June 2004, the risk–benefit evaluation of new cell substrates was judged favourable. WHO was requested to facilitate the development of international consensus on specifications for vaccines produced in cells not currently covered by guidance documents.

The Committee agreed with a proposal that the next steps towards developing consensus among regulators would be for WHO to establish a working group to recommend and coordinate scientific studies to answer specific questions relating to the potential risks from residual cellular DNA and to ensure that safety and broad acceptability of new cell substrates are considered impartially. If warranted by the outcome of the work, a revision of the current document on the use of cell substrates for production of biologicals (WHO Technical Report Series, No. 878) should be proposed.

New vaccine development

A comprehensive list of new vaccines under development was presented, including licensed and candidate rotavirus vaccines; malaria vaccines for which encouraging clinical trial results have been obtained and improved tuberculosis (TB) vaccines based on recent advances in genetics and its application to vaccine developments in general. The use of novel adjuvants was discussed together with the need for generic guidelines for the vaccines being developed for rare infectious agents with pandemic potential. Regarding the preparedness for a pandemic of influenza to which WHO assigns a very high priority, a consensus on how, if needed, to achieve accelerated product characterization, nonclinical and clinical evaluation is a pressing issue.

Regional reference reagents

The Secretariat had identified three models for regional reference reagents and obtained support for the concept from the WHO International Laboratories and Collaborating Centres. A meeting had been held in the South-East Asia Region of WHO to discuss which

reagents should be developed and how they will be deployed. The regional priorities in the South-East Asia Region were identified as regional reference reagents for whole cell pertussis vaccine and inactivated Japanese encephalitis vaccine. The Committee concurred that a mentoring process be used to facilitate the development of such regional reference reagents and that a detailed feasibility assessment of the South-East Asia Region plans be undertaken.

Portfolio for standardization work on vaccines

The Committee was informed that the priorities in the vaccines area over the next few years were revision of existing written standards for rabies, diphtheria, tetanus and pertussis (DTP), bacille Calmette-Guérin (BCG), DNA vaccines, TSE, oral polio vaccine (OPV), IPV and GMP. New written standards will be prepared for live attenuated rotavirus vaccines and regulatory expectations produced for vaccines with pandemic potential. Needs for new reference materials for rotavirus vaccines, malaria vaccines and human papilloma virus vaccines were identified. Future work will be done on the standardization of HIV assays, molecular methods for assuring the safety and efficacy of vaccines and on regulatory expectations for vaccine candidates derived from plants.

Biological therapeutics

In the field of biological therapeutics WHO will enter a new field of work on standardization of cells and tissues for transplantation, and a working group for this purpose is being convened. It is the aim of this group to have a guidance document reviewed by the Committee, if possible at its meeting in 2005. Additional issues related to cytokines and endocrinological substances were covered elsewhere in the agenda.

The Committee considered that the extensive workplan presented fulfilled its needs in a comprehensive way. The Secretariat will set priorities for the programme taking into account the financial and human resources available.

Developments in biological standardization: blood products and related in vitro diagnostics

The Committee was informed about the new commitment of WHO to focus more on country-level activities. Consistent with this is the priority being given in the programme of work to strengthening national regulatory authorities for appropriate regulation of blood and blood products. This is essential because blood can be, and is, a vehicle of transmission of infectious diseases and emerging agents.

It is estimated there are 77 million blood donations per year worldwide of which only 61% result in blood of guaranteed quality. Of the remainder, 34% of donations are collected in countries with deficient regulatory systems leading to elevated risks for recipients, and 5% of blood donations are not tested at all.

The Committee was also informed of the recent and future activities of WHO aimed at improving blood safety on the global level. Several regional workshops and consultations have been held to assist countries to improve blood safety, for example, in Argentina in 2004, for the countries of the Americas on the application of GMP for blood establishments, or in Bangkok, in 2004, on the harmonization of quality assurance (QA) systems for blood products in Asian countries. Thanks were expressed to national regulatory authorities for their support through the secondment of personnel and expertise to support such activities (e.g. the Paul Ehrlich Institute, Germany; Swissmedic, Switzerland; and Center for Biologics Evaluation and Research (CBER), USA).

The formation of regional networks as powerful tools for improving the safety and quality of blood products in the regions, with priority being given to the implementation of GMP rules by blood establishments according to existing guidelines from the Pharmaceutical Inspection Cooperation Scheme (PICS), was under way. Appreciation was expressed for the active involvement of specialized inspectors from PICS in the work. The Committee encouraged WHO to establish an informal group of experts from experienced regulatory authorities to support WHO in this regional work.

One consequence of giving priority to activities for strengthening GMP in blood establishments was that, with the resources available to QSD and consistent with the decision of its fifty-fourth meeting, the revision of the recommendations for blood, blood components and plasma derivatives: collection, processing and quality control remained on hold as an unmet need.

Developments in biological standardization: advancement of technical expertise of regulatory authorities in the area of blood products and in vitro diagnostics

A short overview of the Paul Ehrlich Institute, Germany, and its duties and responsibilities was given. This focused on research in the field of blood products and related in vitro diagnostics. For example, testing of batch release hepatitis B antibody diagnostic kits and HIV antibody diagnostic kits had led to the withdrawal of some products from the market due to a loss of activity documented over time. As

these problems had not been identified by the manufacturers, this emphasized the need for independent testing of key in vitro diagnostic devices by national regulatory authorities. The Paul Ehrlich Institute supports WHO in many respects and proposes to offer its expertise in the field of blood products and in vitro diagnostic devices in particular.

Consistent with the outcome of the International Conference of Drug Regulatory Authorities held in Madrid in 2004, the Committee recommended that WHO promote standards and regulations for blood, blood products and in vitro diagnostic devices, and obtain worldwide cooperation from national regulatory authorities to improve their technical expertise in this area. The Committee agreed that there was a need not only for WHO to build capacity in countries, but also for WHO to facilitate opportunities for cooperative action among experienced blood and blood product regulators. The Committee recommended WHO to establish a global network of experienced regulatory authorities to cooperate in risk assessment and information sharing. The goals of the network may include scientific assessment of current and emerging threats to global public health from blood, assessment of blood-related technologies with an impact on public health, cooperative actions of regulatory authorities, opportunities for regulatory harmonization and advising WHO on those of its activities that have an impact on worldwide regulation of blood, blood products and related in vitro diagnostic devices.

The Committee welcomed the initiative and encouraged WHO to take appropriate steps for its realization the first of which would be the development of terms of reference for the network, which, when formed, should report to the Expert Committee on Biological Standardization.

Developments in biological standardization: new web site for dissemination of information from Quality Assurance and Safety of Plasma Derivatives and Related Substances

A presentation was given on the basis for the establishment of a new web site for the dissemination of information on blood products and regulation of in vitro diagnostic devices to countries worldwide in an efficient and user-friendly manner.

The new web site will, as before, include a page on norms and standards, from which information on WHO International Standards may be obtained. Moreover, in response to the new WHO policy of increased emphasis on countries, four new pages have been created

focusing on strengthening the technical capacity of national regulatory authorities. The new pages are as follows:

Quality and safety of blood products. This page gives an overview of the technical support provided in the area of quality assurance of plasma derivatives and other related substances.

Support to regulatory authorities: This page has introduced a new tool, a basic operational framework (BOF), through which regulatory authorities are able to identify gaps in capacity. An online database is being developed that will make it possible for a BOF questionnaire to be completed online and sent back electronically. The use of BOFs as a web-based tool through the Web for the identification of gaps highlights the interactive nature of the future web site. The activities of the International Conference of Drug Regulatory Authorities (ICDRA) are also included on this page.

Regulation of in vitro diagnostic devices. Links to infectious markers, blood-grouping devices, coagulation disorders and thromboplastin reagents, and to the respective documents from WHO collaborative studies, can be found in this section.

Good manufacturing practices. This page covers activities relating to quality and safety of plasma derivatives and other related substances with information mainly on recent and future workshops.

Additional pages. Pages of topical relevance are also provided: at present these pages cover transmissible spongiform encephalopathies (TSE) and animal sera (which covers antivenom sera). It is intended that new pages dealing with issues of global public health concern will be added as and when the need arises.

The Committee welcomed these improvements and recommended that resources be made available to ensure the development of the web site as an interactive forum.

Developments in biological standardization: reports from the WHO International Laboratories

The Committee was informed of recent developments at the WHO International Laboratories for Biological Standardization.

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

The Committee was provided with an overview from the National Institute for Biological Standards and Control (NIBSC) regarding the production and distribution of WHO International Standards. At the new Centre for Biological Reference Materials at the Institute,

installation and validation of major equipment was in progress. The vial-filling capability was expected to be fully operational by the end of 2004 and the ampoule-filling and sealing machines in the first quarter of 2005. This would substantially increase the production capacity of the Institute. A new project management-based process for developing standards had been established. Information on all projects will be entered in a new database to allow better and longer term resource planning, and to enable filling schedules in the new facility to be optimized. The database is designed to capture all information needed and will allow knowledge to be retained over time. It will give greater transparency to all projects; 67 projects have already been included. The output from the database will be available to the Committee to help in establishing priorities, and a critical element in the development of International Standards will be the endorsement by the Committee of the planned projects. NIBSC welcomed suggestions for improving the database. One point of special interest was the direct accessibility of the database by the Secretariat. Regular contact would be maintained with the Secretariat, especially to discuss new projects.

The Committee was informed of the risks to global biological standardization if the NIBSC were to suffer a catastrophic loss of stocks and of the measures taken by the Institute to militate against this risk. The possibilities for offsite storage of important reference materials have been investigated, and, as a result, it is proposed to store a stock of approximately 100 containers of each International Standard offsite. The major problem in case of a catastrophic loss will be the limited pool of expertise and expert knowledge available to generate full replacement stocks, which means the process would inevitably be lengthy.

The NIBSC offered support for the WHO initiative to establish regional working standards. A short training course was to be offered in 2005. For the future, a more structured course is being planned, but better training facilities at the Institute would be needed for that purpose.

The Committee was reminded of the substantial commitment by the Government of the United Kingdom to biological standardization. However, as a result of a recent review, major management changes are to be implemented, and NIBSC will become the responsibility of the Health Protection Agency of the United Kingdom. There are some concerns for the future, and the Committee considered it essential that the international dimension of NIBSC's work be preserved. At risk is future funding and how this funding will be directed. The

Committee considered it essential for WHO to be actively integrated in the consultation process to enable it to express its concerns.

Feedback from users: issues highlighted by the WHO Global Training Network and by the WHO prequalification process for vaccines

The Committee was informed of the progress in the WHO vaccine pre-qualification process. In April 2004 a consultation was held with the aim of increasing the general understanding of prequalification for vaccines and the involvement of national regulatory authorities in the process, and to adapt the process to respond to new challenges (particularly novel vaccines). The consultation provided valuable feedback to the Committee on the implementation of its guidelines on regulatory expectations for clinical evaluation of vaccines (WHO Technical Report Series, No. 924) after the guidelines had been used during the prequalification assessment of a number of vaccines. It was found that the guidelines defined general principles adequately, but the participants reported a need to develop consensus on certain issues to supplement these general principles. The report of the consultation thus documented examples with specific vaccines as a way of supplementing the general guidance provided in WHO Technical Report Series, No. 924, but considered that there was no need, at present, for the text published in WHO Technical Report Series, No. 924 to be revised.

The consultation also provided feedback to the Committee on the application of the WHO guidelines for GMP for biologicals (WHO Technical Report Series, No. 822). These guidelines are also general and there is a need to harmonize their interpretation by the various GMP experts during WHO prequalification site visits. Different opinions have been expressed particularly regarding the interpretation of guidelines referring to air-handling and quality of water used at different stages of production. Other issues where more guidance would be useful included media fills (a process to check the overall stability of a filling line in the factory), cleaning validation and validation of computerized systems. The consultation requested that the WHO GMP guidelines for biologicals be reviewed, and the Committee agreed with this proposal.

In addition to these matters, the Committee was informed of several changes that will be made to the WHO prequalification process for vaccines, such as streamlining the procedure for site visits if defined criteria are fulfilled, and simplifications of the reassessment procedure.

An essential prerequisite for prequalification is that the national regulatory authority in the country of manufacture is assessed by WHO against six critical functions. These functional indicators were revised during 2004 and the Committee was informed of the new criteria that needed to be met for national regulatory authorities to be regarded as functional by WHO.

Information was given on the activities of the WHO Global Training Network. Since its inception, trainees from more than 80 countries had attended training courses. Training had been conducted for licensing (76 trainees), for clinical trials (53 trainees), for laboratory testing (209 trainees), for lot release (12 trainees), for GMP (205 trainees), and, for recognizing and responding to adverse events after immunization (242 trainees). Depending on future needs, training modules will be developed in other fields such as quality control methods and clinical evaluation. The Global Training Network was also keen to support the proposal from the Committee to develop a training module in biological standardization.

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

Guidelines for production and quality control of candidate tetravalent dengue virus vaccines (live)

In response to expressions of interest from many countries in the development of candidate live attenuated tetravalent dengue vaccines, preliminary draft guidelines on production and quality control specifications for tetravalent dengue vaccine (live) were initially developed by a small drafting group established by WHO. These were presented at the forty-eighth meeting of the WHO Expert Committee on Biological Standardization held in 1997. At that time the Committee advised that further progress in the field of dengue vaccine development was needed to before it would be appropriate for WHO to develop guidance on technical specifications for these candidate vaccines. Subsequently WHO established a Task Force on Clinical Trials of Dengue Vaccines, and at the second meeting of this Group, held in Denver, Colorado, in November 2002, it was considered timely by the experts present, for WHO to recommence the development of the production and quality control guidelines. The WHO Secretariat agreed, and convened a small drafting group to review the previous draft. The drafting group met twice, first in Geneva from 20–21 March 2003, where the original document was reviewed, and then in

Philadelphia, USA, from 2–3 December 2003, when a new draft was reviewed. Taking into account the comments arising from that meeting, document WHO/BS/04.1989 was prepared and distributed for the purpose of inviting comments and suggestions on the proposals therein.

This document covers candidate live attenuated tetravalent dengue vaccines and, moreover, only those vaccine candidates for which active clinical trials are in progress. The WHO Task Force on Clinical Trials for Dengue Vaccines is aware that certain tetravalent vaccine candidates have been generated by taking original patient isolates from each serotype and passaging these isolates in dog primary kidney cells to attenuate the viruses. Extensive testing has been done to define the attenuation phenotype for each of the vaccine candidates. Vaccine formulations are being developed based on the optimal degree of attenuation and immunogenicity. Another type of vaccine candidate has been generated from molecular clones of each of the four dengue virus serotypes. These viruses contains a 30-nucleotide deletion in the 3′ non-coding region that generates candidate attenuated vaccines. In addition, chimeric vaccine candidates that contain the structural region of the other three dengue serotypes in a dengue virus type 4 backbone containing the 30-nucleotide deletion are being prepared for clinical development. A further vaccine candidate is a chimeric vaccine made by expressing the dengue virus structural proteins, prM and E in a molecular clone of the yellow fever virus vaccine 17D backbone. A combination tetravalent vaccine is currently under development, which will have all four dengue serotypes, represented as chimeric dengue–yellow fever vaccines.

Issues that were considered during the consultation process, and by the Committee, included specifications:

— for vaccines developed in Vero cells, dog primary kidney cells and fetal rhesus lung diploid cells;
— for the plaque or focus-forming assay specified for determinations of infectivity in a tetravalent mixture. Because there are as yet no candidate titration standards, WHO was advised to consider developing such reagents and organizing their subsequent characterization by international collaborative study;
— for programmes to determine the thermal stability of the final tetravalent freeze-dried product and the stability of the liquid vaccine after reconstitution. It was noted that in some countries, stability testing of intermediates is required. WHO was requested to develop further guidance on this issue;

— for an accelerated degradation test to be performed on each new batch of vaccine to show the consistency of manufacture of the final stabilized formulation;
— for vectored vaccines where reference to general WHO principles for vaccines for human use derived by molecular methods, that are under development,[1] should be considered; and
— for nonclinical testing of candidate dengue vaccines for which, in addition to the conventional procedures, the issue of antibody-mediated disease enhancement should be addressed.

During the discussion the Committee emphasized that these guidelines covered only candidate vaccines, because no vaccine has been licensed so far.

After making modifications to the document, the Committee recommended that it be adopted and be published as Annex 1 to this report.

Recommendations for the preparation, characterization and establishment of international and other biological reference standards

Biological reference standards form the basis of regulation and clinical dose regimens for biological medicines and also for regulation of in vitro diagnostic devices. The process whereby such international biological reference standards are established and the technical specifications with which they comply are set out in a written standard, which is intended to be scientific and advisory in nature.

WHO written guidance for biological reference standards was first published in 1978, although the first standard preparation was established as early as 1925. The guidance was revised in 1986 following the decisions by the WHO Expert Committee on Biological Standardization to simplify the nomenclature of international biological reference standards and that reference materials of human origin should be tested for evidence of possible contamination. The document was revised again in 1990 when a section was added on information to be provided in support of requests for adoption by the WHO Expert Committee on Biological Standards of international biological reference standards. A number of developments had occurred since then. Partly because of scientific and technical advances, the range of materials classified as biological substances has altered: many older biologicals can now be appropriately characterized by chemical and

[1] WHO Informal Consultation on characterization and quality aspects of vaccines based on live virus vectors. Geneva, 4–5 December, 2003 (available at: www.who.int/biologicals).

physical means and their WHO biological reference materials have been discontinued, while new groups of biological substances have been developed. It has been recognized that there is a need for some reference materials to be made available promptly without the rigorous characterization and testing of international biological standards. This has led to a new group of WHO Reference Reagents which may act as interim standards. A priority-setting process has been published. The science of reference material preparation and characterization has continued to evolve and the extent to which principles for the characterization of reference materials in other fields can be applied to biological reference materials has been debated. WHO has therefore worked with the scientific community, national regulatory authorities, other standard-setting bodies and users through a series of consultations to review the scientific basis of characterization of biological reference materials. As a result, the concepts used by WHO for biological standardization were re-affirmed as being appropriate to ensure the continued usefulness of this class of reference materials. During the consultation process it was recognized that improved clarity in explaining the rationale for the principles used by WHO in biological standardization would be desirable. Accordingly a revised version of the WHO guidance was prepared (WHO/BS/04.1995). This had been reviewed in a consultation held in Geneva from 30 September to 1 October 2004 and an updated document (WHO/BS/04.1995 27 October 2004) was considered by the Committee. After making some changes, the Committee adopted the text as Recommendations for the preparation, characterization and establishment of international and other biological standards and agreed it should be annexed to its report (Annex 2).

The consultative process had also revealed a need for continued scientific and capacity-building work in the area of biological standards. Thus the Committee also recommended that WHO consider starting or continuing work specifically on:

— predicting and monitoring the stability of biologicals;
— specific training modules for biological standardization, with the collaboration of the WHO Global Training Network; and
— developing a manual to describe in detail the calibration procedures for secondary standards.

Recommendations for the production and control of rabies vaccines — proposed revision

The Committee was informed that authoritative information regarding rabies is available in the reports of the WHO Expert Committee on Rabies, most recently in WHO Technical Report Series, No. 824,

published in 1994, which dealt with all issues related to rabies, including immunization schedules, surveillance and epidemiology of the disease.

In the area of vaccine production and quality control, the Committee established the Requirements for rabies vaccine for human use in 1980 (WHO Technical Report Series, No. 658), WHO Requirements for rabies vaccine (inactivated) for human use produced in continuous cell lines (WHO Technical Report Series, No. 760) in 1987, and an amendment which documented the introduction of new reference materials was published in WHO Technical Report Series, No. 840 in 1994. Since that time, significant advances in the production and control of rabies vaccines have been made and the Committee was informed of plans to revise and consolidate the current WHO documents.

In preparation for the above-mentioned revision, a working group on potency assays for rabies vaccines was convened at WHO in Geneva, on 20 May 2003 to review the current approach to potency testing and the recent data generated on in vitro assays. In addition, a drafting group was convened in May 2004 to initiate the revision of the current requirements for rabies vaccines for human use.

The issues that would be considered during the revision were:

— the scope of the document including the substrates for vaccine production that the revised document would cover;
— the inactivation process;
— the test for effective inactivation (following a recent problem with one product);
— potency tests and the use of in vitro assays for determination of the antigen content;
— stability tests and the value of the accelerated degradation test; and
— requirements of national regulatory authorities.

The Committee advised on additional issues to be considered in the revision process. Harmonization between the *European Pharmacopoeia* and WHO requirements with respect to the test for complete inactivation should be achieved. The tests for inactivation currently include a direct test in mice or an indirect test by amplification on cell substrates. The *European Pharmacopoeia* permits the use of immunofluorescence for the detection of virus in cell culture whereas the WHO requirements require a test in mice. The possibility that an analysis of the kinetics of inactivation, which is product- and process-specific, be introduced on a routine basis to monitor the effectiveness of virus inactivation was also suggested for consideration.

Detailed information on the number of doses produced per year and number of doses administered per year in different countries for rabies vaccines produced in different substrates will be useful for the revision of the recommendations. The Committee agreed with a proposal from the drafting group that the production and use of vaccines produced in neural tissues (such as sheep and goat brain) be discontinued and such vaccines should no longer be within the scope of the document. Information concerning obstacles to be overcome in switching to cell culture production, such as difficulty in sourcing a suitable strain of virus for production, the availability of know-how or the cost of production in cell culture and impact on the price of the vaccine should also be investigated.

A difficulty in the area of potency tests (the mouse National Institutes of Health (NIH) test) and the use of in vitro assays for determination of the antigen content (enzyme immunoassays (EIAs) for glycoprotein content) is that there is no correlation between the NIH and EIA test data. Such a correlation was required by the Committee 10 years ago when it rejected proposals for the introduction of tests for glycoprotein antigen content to replace the NIH test in vaccines of proven consistent production. At the meeting of the drafting group in May 2004, it was agreed that assays for glycoprotein antigen content may demonstrate consistency of production. The group considered the available reagents and whether monoclonal antibodies could differentiate between antigenic but nonimmunogenic vaccines. Reagents that react only with conformational epitopes found on immunogenic antigen are available. It was suggested that EIA as a measurement of consistency of production could be used together with the NIH test for a period of time. After a review of data generated from tests in parallel, the omission of the NIH test could be considered for an individual vaccine. Moreover, it was proposed that the performance of a single-dose mouse protection test be recommended for vaccines for which the parameters of the NIH tests have been established with well-established vaccines.

The Committee concluded that the scope of the revised recommendations should include inactivated vaccines produced in cell cultures, ranging from primary cells (hamster and chick embryo), human and monkey diploid cells, to continuous cell lines, and also vaccines containing inactivated virus purified from duck embryos. Furthermore the Committee suggested that the requirements for rabies vaccines for human use (WHO Technical Report Series, No. 658) that cover the production of vaccines in neural tissue should not be discontinued immediately once the revised requirements for rabies vaccine are accepted, but should remain valid for a defined but short

period (e.g. 3 years) to allow manufacturers to switch to alternative substrates.

Recommendations for the production and quality control of diphtheria, pertussis and tetanus vaccines — proposed revision

Outcome of the meeting of the European Directorate for the Quality of Medicines on serological potency tests for diphtheria and other vaccines

The outcome of the meeting on serological potency tests for diphtheria and other vaccines held in October 2004, which was attended by 57 participants from 17 countries, was presented to the Expert Committee on Biological Standardization. The report will be published in *Pharmeuropa*, 2005, **17**. The participants at the meeting considered the implications of a recently completed international collaborative study of serological potency tests for diphtheria-containing vaccines. The study confirmed that serological potency tests were suitable for assay of a wide range of diphtheria-containing combination vaccines that are on the market in Europe. As a next step, EDQM will propose a modification of the *European Pharmacopoeia* to introduce such tests in addition to the currently used lethal challenge test. This would bring the *European Pharmacopoeia* into line with WHO specifications which have allowed the possibility of such tests for more than 10 years. Guidelines will be established regarding validation and implementation of the new method. Training courses and access to standards will be organized.

Preliminary data presented at the EDQM meeting suggested that it may be possible to select appropriate dilutions of vaccine and reference antigen to inoculate into animals so that the serological method may be used to assay responses to diphtheria and tetanus simultaneously, and possibly also to IPV. Also a one-dilution assay in guinea-pigs was discussed for routine batch release by manufacturers and official medicines control laboratories.

Proposed revision of WHO recommendations

The Committee was informed about a planned revision of the WHO Requirements for diphtheria, tetanus and pertussis vaccines (WHO Technical Report Series, No. 800). An amendment to this document, endorsed by the Committee in 2003, considered only some aspects of the potency tests for diphtheria and tetanus vaccines. This amendment was considered as a first step towards a full revision with the objective of achieving further harmonization. A number of issues for consideration during the revision process had been identified, espe-

cially concerning pertussis vaccines. For whole-cell pertussis vaccines, these included the assay of bacterial concentration; evaluation of production consistency; specifications for the potency test; the scientific basis for the mouse weight-gain test; and characterization of the fourth International Standard. For acellular pertussis vaccines, the issues included specifications for potency and toxicity tests; the extent of nonclinical testing, and the design of clinical trials, required for a new manufacturer of acellular pertussis vaccine; and the duration of protection. The Committee was reminded that it had published a separate document to give guidance on acellular pertussis vaccines (WHO Technical Report Series, No. 878), and was requested to give guidance on whether to continue with two different documents.

The Committee advised that the goal should be to have one document on diphtheria, tetanus and pertussis with separate sections on whole-cell pertussis vaccine and acellular pertussis vaccine. The first step towards achieving this should be updating the Requirements for diphtheria, tetanus, pertussis and combined vaccines (WHO Technical Report Series, No. 800, Annex 2). The existing guidelines for acellular pertussis (WHO Technical Report Series, No. 878) should be updated as a distinct step but, once finalized, both sets of revised recommendations should be published together in one volume of the WHO Technical Report Series.

Guidelines for the safe production of poliomyelitis vaccines from attenuated Sabin strains — proposal

The current status of the global polio eradication programme was described to the Committee. In 2003, only 784 cases had occurred in six countries. However, starting in 2003, vaccination with the oral poliomyelitis vaccine (OPV) was completely stopped in one region of one of the countries in which polio remained endemic. Vaccine was withheld for more than a year and, as a result, poliovirus reseeded not only this country but also other countries in the region, including countries that had been completely polio-free in recent years.

The strategic goals were reaffirmed, i.e.

— that there would be no more polio cases worldwide after 2005; and
— cessation of vaccination with OPV after a suitable interval of time following the last case of polio, in all countries of the world.

It was considered likely that a number of countries would change to vaccination with IPV. The manufacture of IPV requires the production of large volumes and high concentrations of virulent live wild-type polioviruses that are then inactivated. Manufacture is currently limited to producers in Canada and Europe. The Committee had

previously established guidelines for the safe production and quality control of IPV to manage the risk of reintroduction of wild polioviruses from production facilities when wild polioviruses no longer circulate in the community. At an ad hoc Advisory Committee on Polio Eradication (AACPE), meeting held in 2004, a programme of work for cessation of OPV in the future was established. In the context of biological standardization, there was a need to refine the current guidelines on the safe production and quality control of IPV, to define measures for the containment of the Sabin strains at production sites after cessation of OPV use, and to establish effective tools to verify the implementation of biosafety guidelines on manufacturing sites.

The Committee was informed that monovalent OPV (mOPV) is intended for a WHO stockpile in case an emergency need for vaccination should occur after all vaccination with OPV has ceased. mOPV induces type-specific mucosal immunity more rapidly than do the trivalent vaccines, leading to a faster specific protection in the population. The AACPE advised that mOPV 1 may be used before polio had been eradicated in a country or region where only wild poliovirus type 1 circulated. It was anticipated that mOPV 1 may be introduced into one country in 2005, and it was noted that the registration of mOPV 1 for the eradication of wild poliovirus type 1 would be beneficial not only in accelerating the eradication of wild poliovirus in that country, but also to gain up-to-date experience in the use of the future stockpile vaccine.

Another high priority is the development of Sabin derived IPV vaccines as an alternative to the wild-type derived products used today. This will have implications for the written standards and reference standards produced by WHO. The Committee advised on additional items of importance, namely how to maintain the expertise in polio neurovirulence testing for the future, and recommended that WHO determine whether at least one centre should be designated for that purpose.

The Committee agreed that this programme be further developed and encouraged rapid development of the proposed written standards and reference standards.

Recommendations, guidelines and other documents for biological substances used in medicine: review of the consolidated list

The recommendations (formerly "requirements") and guidelines published by WHO are scientific and advisory in nature, but they may

be adopted by a national regulatory authority as national requirements or they may be used as the basis for national requirements. These international recommendations and guidelines are also 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 testing, assay and control to ensure the quality, safety and efficacy of these products.

A consolidated list of WHO Recommendations and Guidelines, together with a list of a variety of other documents produced by the WHO biological standardization programme, is published as an Annex to each report of the ECBS (the latest version appeared as Annex 7 to the fifty-fourth report of the Expert Committee on Biological Standardization). No comprehensive review of the items in the published list has been performed in recent years. At the request of the Secretariat, the list of recommendations and guidelines for biological substances used in medicine and other documents was reviewed. Proposals for changes to the format of the list of Recommendations, Guidelines and other Documents and individual items for revision, updating or possible discontinuation were made (WHO/BS/04.1996).

The Committee identified a number of documents that could be discontinued. They decided to follow the procedure established at its forty-eighth meeting according to which proposals to discontinue a WHO standard were published for comment before a final decision was taken. The Committee recommended discontinuation of the following documents in 2005, provided that no objections were raised:

— Recommendations on Human Interferons made by Recombinant DNA Techniques (WHO Technical Report Series, No. 771, 1988), because the principles are covered by the general Recommendation on Products made by Recombinant Techniques (WHO Technical Report Series, No. 814, 1991).
— Rift Valley Fever Vaccine (WHO Technical Report Series, No. 673, 1982)
— Development of national assay services for hormones and other substances in community health care (WHO Technical Report Series, No. 565, 1975)
— Guidelines for quality assessment of antitumour antibiotics (WHO Technical Report Series, No. 658, 1981)
— Recommendations for the assessment of binding-assay systems (WHO Technical Report Series, No. 565, 1975)
— Summary protocol for the batch release of virus vaccines (WHO Technical Report Series, No. 822, 1992)

The Committee also noted that a number of reports from WHO informal consultations were listed in the Annex published after each report of the Committee, but that this was not done in a consistent way. The Committee advised that such reports should be systematically listed, and made available through the web site. In view of this decision the following reports will no longer be listed in the Annex published in the WHO Technical Report Series.

— Standardization of interferons (WHO Technical Report Series, No. 687, 1983; 725, 1985; and 771, 1988)
— Report on the standardization and calibration of cytokine assays (WHO Technical Report Series, No. 889, 1997)

The Committee further noted that some of the documents need revision because the types of vaccines used have changed (e.g. typhoid vaccines and rabies vaccines). Where work was not already in progress, the Committee advised the Secretariat to review the need and to develop a plan to address this. The Committee endorsed the conclusion of the review that the publication of a single volume containing all the updated recommendations and guidelines should be considered. Such a volume would collect together into one place documents appearing in many different reports of the Committee and constitute an important resource for all those involved in the development, manufacture and regulation of biologicals. The list should also be made available on the web site.

Finally, the Committee advised that the list should be regularly reviewed to identify other biological products or topics of emerging importance for which the drafting of recommendations or guidelines would be beneficial.

After making the changes noted above, the Committee recommended that the list be adopted and be published as Annex 3 to this report.

Quality, safety and efficacy of antivenom sera

There are approximately 3000 species of snake worldwide, 600 of which are venomous. Studies carried out over the last 60 years have shown high incidences of snake bites leading to numerous deaths in humans (global estimate: 50 000 to 125 000 deaths per year). Not all snakebites are fatal, but may result in permanent physical disability (including the need for limb amputation), chronic ulceration, osteomyelitis with malignant transformation, chronic renal failure, chronic pituitary-adrenal insufficiency and neurological sequelae.

Antivenom therapy is key to the medical management of snakebite and other venomous bites and stings. Unfortunately there are a number of problems for developing countries in assessing and using antivenoms. These include:

— a reduction in the number of manufacturers;
— poor regulatory control over the manufacture of antivenoms;
— poor regulatory control over their importation;
— high costs;
— logistical problems in distribution; and
— lack of knowledge about the correct medical management of bites and stings from venomous animals, including the appropriate use of antivenoms.

The collaboration of manufacturers and regulatory bodies worldwide is needed to ensure the availability of safe and effective antivenoms.

The Committee welcomed this item being brought up again and strongly recommended WHO to reconsider better resourcing of the respective units at WHO and to strengthen the technical capacity of regulatory authorities and the formation of regional networks to improve regulatory control over the manufacture, import and sale of antivenoms. Attention should be given to providing better education in countries to improve prevention of snakebites and to ensure best practice in the use of antivenoms. In addition initiatives should be taken to obtain additional funding to sustain the manufacture of these products.

Good manufacturing practices for blood establishments: progress report on training activities

The Committee was informed about the activities of WHO regarding the application of GMP, which is one of the most productive areas for improving the safety of blood and blood products in the regions. The major problems in many countries are:

— lack of regulation;
— regulation that is inadequate or not implemented;
— lack of government awareness; and
— lack of understanding and technical capacity.

An important goal is to bring together all parties involved: the regulatory authority, inspectors, plasma supplier and plasma fractionators, and blood transfusion institutions. To start the implementation of GMP, the training of inspectors to give them sufficient knowledge of blood collection and plasma fractionation is a key factor.

The experience gained from the first training course held in Argentina in 2004 was presented. Eight countries participated. On-site inspections were performed during the workshop. The PICS Guide was accepted by all the countries as their future standard. Regional specifications may need to be added to this general GMP guide.

The Committee expressed its appreciation of the efforts made in improving blood safety as this was work of the utmost importance, and encouraged WHO to continue.

International Reference Materials

Comparison of glass ampoules versus rubber-stoppered vials for the storage of international biological standards

The Committee was informed of the outcome of a study performed at NIBSC to compare the appropriateness of ampoules with that of vials for storing international biological reference standards (WHO/BS/04.2004). The current preference is to use ampoules which are thought to offer superior long-term stability. Working standards in the pharmaceutical industry, by contrast, are often stored in stoppered and screw-capped vials. In a comparative study, a physicochemical evaluation of lyophilized albumin stored in stoppered vials, screw-capped vials and conventional ampoules was undertaken. Low-temperature freeze-drying was used for all three types of container. Determinations of residual moisture using Karl Fischer titration and of residual oxygen using Orbisphere equipment were made over time and at different temperatures. A statistical analysis of the data obtained showed that moisture in ampoules did not change over time, whereas it increased in screw-capped and stoppered vials. This increase occurred in the first 2 months, after which it tended to stabilize. It is assumed that the moisture was derived from the stoppers. Although the oxygen content in ampoules showed higher starting levels there was no variation over 12 months of storage time. Because moisture levels in ampoules do not vary over time and gas levels stay stable, it was concluded that heat-fused ampoules are preferable to vials for the storage of reference materials which are intended for indefinite storage and for which stability is an essential requirement. The Committee concurred that such containers should be the default option for WHO international biological reference standards. However, it was recognized that other factors may influence the choice of container, especially for infectious fills or for working standards, and the Committee recommended that decisions on alternative closures be made on a case-by-case basis.

Priorities for replacement and new international biological reference standards for biologicals

National Institute for Biological Standards and Control

Current projects on international standardization were presented to the Committee as laid down in the new database at NIBSC. There are 67 projects included, of which more than 40 deal with viral or bacterial vaccines. The current list should be used by the Committee as the starting point for its work and will be updated continuously. NIBSC welcomes any suggestions for improving the database. The projects on the list were considered as approved.

The Committee provided advice on a number of specific projects on bacterial vaccine standardization at NIBSC as described below.

Standards for Haemophilus influenzae *type b vaccine*

Quality control testing of *H. Influenzae* type b conjugate vaccines depends to a great extent on physicochemical methods to ensure the consistency of manufacture of batches. A wide variety of chemical assays are used for quantification of 3-B-D ribofuranosyl(1-1)-D-ribitol-5-phosphate (referred to as PRP), the units of which the linear polymer of the type b polysaccharide is comprised. The assays for PRP are performed at various stages of manufacture: on the purified polysaccharide; after it has been covalently coupled to the protein carrier; on the bulk conjugates; and on the final lot vaccines. All assays assign the quantity of PRP in absolute values (μg/ml).

A candidate PRP reference preparation containing a known quantity of polysaccharide had been prepared to facilitate calibration of the assays. This candidate PRP standard was the subject of a collaborative study for evaluation.

The Committee expressed its opinion that the proposed use of the material should be clarified and defined, and advised that if it was intended to assign a unitage in SI units (μg/ml) to this preparation then the specifications in the newly established WHO Recommendations for preparation, characterization and establishment of international and other biological reference standards (Annex 2) should be adhered to.

Progress with replacement of flocculation standards for diphtheria and tetanus

The first international reference reagent for diphtheria toxoid for flocculation test and the first international reference reagent for tetanus toxoid for flocculation test were established in 1988 for use in calculation of the "limit of flocculation" (Lf) units and confirmation of

antigenic purity. The flocculation test is still the method of choice of manufacturers to establish the antigenic strength of toxoids although other methods can be used to confirm purity and consistency of production. The Committee agreed at its 2003 meeting to initiate a collaborative study to prepare suitable replacement reagents. The Committee was informed that, in feasibility tests, the candidate replacement reagent for tetanus toxoid preparations was found to be unsuitable for use in the flocculation test because of a high glycine content. An alternative material for use in the flocculation test has been secured from the Statens Serum Institute in Copenhagen and trial formulations are being tested at NIBSC. Assuming that the new candidate is of appropriate quality, material would be distributed for a collaborative study for which completion and analysis were scheduled for the end of 2005. The candidate replacement material for diphtheria flocculation tests was confirmed as suitable in feasibility tests, and collaborative studies of this material are also planned.

Sanquin-CLB

The Committee was informed that there was a need for thromboplastin standards to be replaced because of reduced stocks, and that it was not yet clear if, in the future strategy at Sanquin-CLB, international biological standardization activities for WHO would still be given high priority. The Committee recommended that continuity of the custodianship of WHO standards and support for work in this area of biological standardization was of the utmost importance and the Secretariat was requested to explore ways to ensure continuity of custodianship.

European Directorate for the Quality of Medicines

The Committee was informed that more than 50% of all projects currently being undertaken by the European Directorate for the Quality of Medicines (EDQM), to establish regional reference reagents are done in collaboration with WHO, and also the US Food and Drug Administration, to make most efficient use of resources and time. A major problem facing European laboratories, and which will have repercussions worldwide, is that the manufacturer of the hepatitis B in vitro assay kit used in vaccine potency tests will cease production in the near future. A replacement assay is being studied with the support of the EDQM. A smallpox immunoglobulin standard would be established by EDQM towards the end of 2004. The Committee was further informed that the EDQM would take the lead in developing mycoplasma reference materials within the International Conference on Harmonisation (ICH) process. Programmes are under

way for blood products such as a working standard for Factor VII concentrate and normal human plasma for assay of solvent/detergent plasma. Replacement methods are being developed for biotechnology products such as erythropoietin. A clear need for a reference for botulinum toxin was identified. Problems have been identified in assays with von Willebrand factor and the suitability of the current standard was under review. EDQM had started to develop methods in the clinical field such as neutralization tests for unwanted antibodies to biological therapeutics and to evaluate, if possible, a single standard method for the evaluation of immune responses to vaccine candidates in the case of a pandemic of influenza. A wish was expressed for the EDQM and the Committee to collaborate in the most efficient way possible.

Proposed disestablishment of the International Reference Reagent for hepatitis B vaccine

The International Reference Reagent, NIBSC code 85/65, for plasma derived hepatitis B vaccine was established in 1987 (WHO Technical Report Series, No. 760). This reference reagent has no assigned unitage and, although the potency of successive production batches of a given product should give consistent potencies relative to this material, this material cannot be used for the establishment and calibration of secondary standards. There has never been a minimum potency requirement based on the use of this material. Furthermore, it has always been emphasized that hepatitis B vaccines which are suitable for use in humans need not be qualitatively equivalent to this reference reagent. Despite this, some vaccine manufacturers have promoted the use of their vaccine by saying that its potency is as good or better than the International Reference Reagent.

The International Reference Reagent no longer serves a useful function. Therefore the Committee agreed with a proposal (WHO/BS/04.1991) that the International Reference Reagent for hepatitis B vaccine be discontinued.

Antigens and related substances

Smallpox vaccines — progress report on proposed second International Standard

Smallpox as an endemic infection was officially declared eradicated by WHO in 1980. Since that time the usage and production of smallpox vaccine, of which vaccinia virus is the active component, has dramatically declined. However, the fact that reference stocks of smallpox

virus were kept after its official eradication and the possibility that it may emerge as a weapon of bioterrorism has led many governments to retain vaccine stocks for emergency use. In the light of recent world events, many governments have reassessed their reserves of smallpox vaccine and in some cases are promoting renewed production and licensing of the vaccine. Although the re-establishment of vaccine production is technically feasible and is under way in some countries, suitable reference materials for the standardization and control of smallpox vaccines are now in short supply.

The first International Reference Preparation for smallpox vaccine was established in 1963 from the Lister strain of vaccinia virus produced on the flanks of sheep. At the time that the International Reference Preparation was produced, cell-culture systems were much less reliable than they are today. Studies conducted at NIBSC using cell-culture plaque assays to assay vaccine materials also tested in chorioallantoic membrane assays in eggs have indicated much greater sensitivity for the cell culture methods, which, if properly validated, could be acceptable assays for potency.

At the fifty-fourth meeting of the Committee, the report of a collaborative study on the suitability of candidates to replace the current International Reference Preparation (WHO/BS/03.1977) had been evaluated. Two suitable candidate preparations had been identified for use as replacements. However, consistent with the proposal from the study participants, the Committee noted that the current International Reference Preparation still has acceptable potency and that as existing stocks of the current preparation are sufficient for the time being, its replacement was not urgent. The Committee thus agreed to defer any decision about replacement pending generation of further information, including stability data and information about rate of supply. Stability studies had been carried out on the two candidate replacements (WHO/BS/04.1990). Stability at the recommended storage temperature (–20 °C) was good for both candidates and for the current International Reference Preparation — showing no loss in activity over a period of 591 days. At higher temperatures, 37 °C and 60 °C, both candidates showed equivalent losses of activity over a given time period, although both appeared to be less stable than the current International Reference Preparation. At 4 °C there were small but consistent losses in activity after storage for periods of up to 280 days.

At the time of reporting, stocks of the International Reference Preparation stood at 375 ampoules. Usage over the preceding 18 months had been very slow; no samples had been requested since April 2004.

Therefore, even with a final archive of 100 ampoules to be retained, there was still a sufficient stock of the current International Reference Preparation to last for 5–6 years at the maximal usage rate observed over the past 3–4 years.

The stability data generated in the collaborative study have indicated that there may be differences between the candidate replacements for the current International Reference Preparation in terms of their thermostability. Therefore the Committee decided that the current International Reference Preparation be retained for at least another 12 months and recommended that the stability study on the candidates be extended to include assays in chorioallantoic membrane in addition to cell culture assays if possible, and that the data be reviewed for discussion at the meeting of the Expert Committee on Biological Standardization in 2005. The Committee did not agree with a proposal to re-assign the potency value of the current International Reference Preparation.

Yellow fever vaccine — outcome of an enquiry regarding the use of the first International Standard

The collaborative study that established the suitability of the first International Standard for Yellow Fever Vaccine indicated that the use of the standard, which has been assigned a potency of $10^{4.5}$ International Units (IU) per ampoule, would markedly improve agreement in results between laboratories.

The WHO requirements for potency of yellow fever vaccine require that the titre of the vaccine should not be less than 1000 times the dose that will kill 50% of mice (1000 LD_{50}) or its equivalent in plaque-forming units (PFU), in the dose recommended by the manufacturer for use in humans. Each laboratory involved in testing the vaccine potency should establish the relationship between mouse LD_{50} and PFU potency. In some cases this was done many years ago and the relationship may not necessarily be valid today.

An additional aim of the original study was to facilitate the replacement of the expression of vaccine potency, currently mouse LD_{50}, with IU determined in plaque assays. It appeared from the correlation curve established from the study data that a minimum potency determined in plaque assays of $4.0 \log_{10}$ IU/0.5 ml relative to the candidate standard would be equivalent to $3.0 \log_{10}$ mouse LD_{50}/0.5 ml, based on the overall means of all laboratory results. However it was acknowledged that this would have to be confirmed in a larger number of plaque assays in which a standard calibrated against the first International Standard 99/616 is included.

The aim of the current study (WHO/BS/04.1993) was to determine whether the use of a minimum potency of 4.0 $\log_{10}$ IU/0.5 ml dose was suitable as the minimum potency for yellow fever vaccines assayed in plaque assays.

Eight participants submitted data from a total of 102 routine production batches of vaccine. The data indicated that some of the vaccines tested would have potencies less than 4.0 $\log_{10}$ IU/dose. All of these vaccines currently meet the minimum WHO requirement of 1000 LD_{50} when plaque titres were converted to LD_{50} equivalents. Some products also had values of less than 4.0 $\log_{10}$ IU after heating in the accelerated degradation test.

The Committee was informed that additional data on vaccine batches stored at the recommended temperature, and on vaccine tested for stability would be collected over the following months. Manufacturers would also be asked for any clinical trial data and information on the minimum PFU or LD_{50} which results in seroconversion. Proposals for the revision of the minimum potency requirements for yellow fever vaccines would be submitted after consideration of these additional data.

Poliomyelitis vaccine, oral — second International Standard

Oral polio vaccine plays a pivotal role in the Global Polio Eradication Programme and will continue to do so until eradication is complete. Although the eradication programme is in its final phase the remaining "hot spots" of endemic poliovirus circulation require large amounts of OPV in the short to medium term. In the medium to long term after eradication, there will also be a need to maintain stockpiles of the vaccine to deal with any unexpected re-emergence of the virus.

The manufacture and control of this vaccine must therefore be maintained at the highest level to ensure that eradication succeeds, and suitable standard preparations must therefore be available to ensure that testing meets appropriate regulatory requirements.

The first International Standard for the potency estimation of OPV was established in 1995. It has been used for the calibration of regional working references and the in-house references of a number of manufacturers and national control laboratories. The stocks of this standard (NIBSC reference 85/569) are now very low and it is imperative that a replacement be established soon to meet the demands of the final stages of the polio eradication programme.

The potency of a trivalent OPV preparation as a candidate replacement for the current International Standard was assessed by

a collaborative study (WHO/BS/04.1992). Thirteen laboratories participated in the study, drawn from five OPV manufacturers and eight national control authorities. Five samples were assessed in the study; the candidate preparation was tested as a coded duplicate sample. Laboratories were requested to use their own antisera for neutralization in the test (of which nine elected to do so), but were also asked to include monoclonal antibodies from NIBSC, which were available as freeze-dried preparations. The overall levels of within-assay variability and within laboratory variation indicated, as in previous studies, a high level of consistency within laboratories. For all poliovirus types in all study samples the values obtained from the different laboratories were all within 0.5 $\log_{10}$ tissue culture infectious dose $(TCID)_{50}$ of the mean — indicating good consistency between laboratories. There were no problems encountered in the use of the candidate second International Standard and all other samples behaved as predicted.

The candidate second International Standard was produced at NIBSC from three commercially produced monovalent bulks — one of each poliovirus (Sabin) types 1, 2 and 3. The passage level of the virus in the bulks is: Sabin original (SO) + 3 for type 1, SO + 3 for type 2 and a re-derived SO (RSO) + 3 for type 3. All three bulks used in the production of this standard were produced on primary monkey cells. Each of the monovalent bulks had previously been released by a national control laboratory and can therefore be considered to be in compliance with current licensing, pharmacopoeial and WHO requirements.

The candidate standard is expected to show at least the same stability as the current standard i.e. no loss of activity over 19 years of storage at –70 °C.

The Committee recommended the establishment of the candidate 02/306 as the second International Standard for the potency testing of trivalent OPV. The recommended potency for this preparation was assigned as 7.51, 6.51, 6.87 and 7.66 $\log_{10}$ $TCID_{50}$/ml for type 1, 2, 3 and total virus content respectively.

Diphtheria toxin: proposed new use for an International Standard

At its meeting in 2003 the Committee agreed that a reference diphtheria toxin standard was required. The WHO International Standard for Schick-test toxin (STT) was established in 1955 (WHO Technical Report Series, No. 96). The intended use at that time was for determination of immune status to diphtheria by intradermal challenge. The

need identified by the Committee in 2003 was for a standard for use in cell-culture assays. The Committee was informed at the current meeting that the preparation of a new, stable, freeze-dried WHO reference standard for diphtheria toxin would require considerable time and effort. The Committee was further informed that recent studies have shown that STT had a defined diphtheria toxin activity, and high real-time stability, with a toxin activity almost unchanged from that recorded when it was originally made in the 1950s. The Committee was therefore invited to consider a proposal (WHO/BS/04.2001) to re-assign the use of STT diphtheria toxin to include use in cell-culture assays for diphtheria toxin.

Cell-culture assay (Vero cell) is now an established method to confirm the presence of diphtheria toxin, or conversely to confirm the absence of toxin or to confirm the freedom from toxin reversion for toxoids. A condition for use of the assay is that the sensitivity of the test must be demonstrated to be not less than that of the guinea-pig test. Because it is not possible to monitor the sensitivity of the cell-culture assay by standardizing the assay conditions alone, the use of an appropriate reference standard of known toxin activity and stability is essential.

The Committee was also informed that STT toxin had recently been included in a collaborative study that led to the establishment of the first European Pharmacopoeia Biological Reference Preparation (EP BRP) for Diphtheria Toxin to be used in cell-based assays of toxicity. The toxin activity of the EP BRP and of STT formulations was studied using the guinea-pig subcutaneous (lethal) and intradermal (non-lethal) assays, as well as the in vitro Vero cell test.

The Committee noted however that STT was of relatively low toxicity and did not meet the criteria for toxicity for Vero cells set by one regional pharmacopoeia. The Committee also expressed concern that contaminants in the preparation may influence the outcome of the cell culture toxicity test. The Committee requested further information on how these factors influenced the suitability of STT for the intended new use. The Committee therefore recommended that the proposal be deferred until further information was available, but nevertheless reaffirmed the need for such a standard and requested that, in addition to the reappraisal of STT, the possibility of making the EP BRP available as an interim international reference reagent be investigated.

Pertussis vaccine, whole cell — progress report on proposed fourth International Standard

Although whole-cell pertussis vaccine has been largely replaced by acellular pertussis vaccines in Japan, North America and Western Europe, whole-cell pertussis vaccine is still widely used in many developing countries and in some countries in Eastern Europe. Therefore an international standard for whole cell pertussis vaccine was still needed and was likely to be necessary for a considerable time.

The current standard is the Third International Standard (ampoule code 66/303) which was established in 1998 (WHO Technical Report Series, No. 897). This material was originally prepared from the same bulk material as the second International Standard, with which it was included in a collaborative study, and its stability was confirmed by subsequent study before its establishment in 1998.

A planned candidate replacement standard (ampoule code 94/532) is currently available, and a collaborative study to compare this candidate with the Second International Standard (ampoule code 66/302) was organized by the Statens Serum Institute, Copenhagen, Denmark and carried out in 1995–1996, together with some preliminary stability testing. In 1998, the remaining ampoules of this material (2790 ampoules) were transferred to NIBSC together with the data from this study.

However, in consideration of the requirement that the potency of the preparation coded 94/532 should be checked in terms of the current third International Standard to confirm continuity of unitage, the Committee was asked to comment on a proposal for a further small-scale study of the stability of the candidate replacement standard and comparison of the candidate replacement standard with the current third International Standard. The Committee agreed with this proposal. Moreover, they requested that the International Laboratory for Biological Standards investigate the feasibility of combining such a study with a recently proposed study to evaluate the suitability of a new regional reference standard to be developed in the WHO South-East Asia Region.

Anti-pertussis typing-sera: WHO reference reagents for serotypes 2 and 3

WHO has recommended that whole cell *Bordetella pertussis* vaccines should contain strains expressing agglutinogen 2 (equivalent to Fim 2) and agglutinogen 3 (equivalent to Fim 3) (WHO Technical Report Series, No. 800, 1990). The identity of agglutinogen 1 is not known,

but it is not appropriate for discriminating between *B. pertussis* isolates as it does not vary. Monitoring of fimbrial expression is recommended not only as a simple means for detecting changes in *B. pertussis* populations, but also because fimbriae could be important protective antigens.

Serotyping is one of the traditional methods used for typing *B. pertussis* strains and serotyping with polyclonal antisera has been used routinely for many years. However, comparison of serotyping results between laboratories is difficult because of the different sera and assays used. The outcome of a meeting held in May 1999 of laboratories involved in epidemiological research on pertussis strains suggested that it would be sensible to use monoclonal antibodies for typing fimbriae.

The Committee was informed that preparations containing monoclonal antibodies to *B. pertussis* serotype 2 (coded 04/154) and serotype 3 fimbriae (coded 04/156) which are intended to be used for both serotyping of clinical isolate strains and for monitoring the consistency of pertussis vaccine production, have been obtained. They were assessed using a standard pertussis strain panel in a collaborative study by 11 laboratories in nine countries for their suitability to serve as reference reagents for *B. pertussis* serotyping in two major methods, namely microplate agglutination and slide agglutination (WHO/BS/04.1998). Rabbit polyclonal antibodies to fim 2 (coded 89/598) and fim 3 (coded 89/600) were included as controls as these polyclonal antibodies have been in use since 1989 as typing reagents for *B. pertussis* serotype in the slide agglutination method. In parallel, monoclonal antibodies produced from a second source of hybridoma cell lines were also compared with preparations 04/154 and 04/156. Under the recommended assay conditions, there was close concordance between the results obtained by the different laboratories using either method. Preparations 04/154 and 04/156 showed good specificity in both typing methods (>90% sensitivity on the homologous strains and <0.1% cross-reactivity). Monoclonal antibodies obtained from the second source showed less sensitivity than preparations 04/154 and 04/156.

On the basis of the results of this study, the Committee endorsed establishment of the preparation of monoclonal antibody to fimbriae 2 (04/154) as the WHO Reference Reagent for *B. pertussis* serotype 2 and monoclonal antibody to fimbriae 3 (04/156) as the WHO Reference Reagent for *B. pertussis* serotype 3. It was noted that freeze-dried preparations were also under development and that the suitability of these preparations as future International Standards would be evaluated.

Blood products and related substances

Anti-A and anti-B blood typing serum: proposed reference reagents

The quality of blood grouping reagents is clearly an important factor in ensuring safe blood transfusion, yet there is currently no appropriate international standardization of anti-A or anti-B blood grouping reagents. Suitable international reference reagents are needed to ensure minimum standards of potency for such reagents. Although WHO reference standards exist, a WHO consultation in 1999 concluded that they were not relevant to the methods currently used by immunohaematology laboratories. Accordingly, the preparation and characterization of monoclonal antibody blood grouping reagents was initiated by WHO for the development of new reference materials.

A collaborative study to evaluate lyophilized monoclonal IgM anti-A (code 03/188) and anti-B (code 03/164) preparations to determine an appropriate dilution of the reconstituted contents to specify the minimum acceptable potency of anti-A and anti-B blood grouping reagents was reported to the Committee (WHO/BS/04.1999Add1). The candidate reagents were evaluated against a wide range of commercial anti-A and anti-B blood grouping reagents in an international collaborative study involving 16 laboratories in nine countries. Data were received from all 16 laboratories. These laboratories tested 23 different anti-A reagents, together with the candidate reference reagent 03/188, and 25 different anti-B reagents together with the candidate reference reagent 03/0164.

Considerable variations in haemagglutination end-point titres for anti-A and anti-B activity were found between reagents and between laboratories. Although the titres for each of the candidate reference reagents showed less variation within a phenotype, the results showed that, even when nominally using the same method, there was wide variation between laboratories in the sensitivity of the haemagglutination tests.

Most anti-A reagents would meet a minimum potency specification of an eightfold dilution of reagent 03/188; most anti-B reagents would meet a minimum potency specification of a fourfold dilution of reagent 03/164. However, adoption of these specifications might encourage many manufacturers to dilute their reagents more than they do at present. Minimum potency specifications corresponding to a four- or fivefold dilution of reconstituted reagent 03/188 and a two- or threefold dilution of reagent 03/164 would be more in line with the current quality of most anti-A and anti-B reagents, respectively,

tested. International consensus on assignment of the most appropriate dilution for 03/188 and 03/164 could not be reached by the study participants.

The Committee took note of the report. Agreement on the value assignment had however to be reached before the reagents could be endorsed as WHO standard reagents. The study was referred to a working group for this purpose. Advice on the impact of the methodology used in the study would also be requested from the working group, together with advice on revisions to the study report.

Anti-D blood typing serum: first International Standard for minimum potency of blood grouping reagents

The quality of blood-grouping reagents is clearly an important factor in ensuring safe blood transfusion, yet there is currently no appropriate international standardization of anti-D blood-grouping reagents. Suitable international reference reagents are needed to ensure minimum standards of potency of such reagents. Although a WHO reference standard exists, a WHO consultation in 1999 concluded that it was not relevant to the methods currently used by immunohaematology laboratories. Accordingly the preparation and characterization of a monoclonal antibody blood-grouping reagent was initiated by WHO for the development of a new reference material.

A collaborative study evaluated a lyophilized monoclonal IgM anti-D preparation (code 99/836) to determine an appropriate dilution of the reconstituted contents to specify the minimum acceptable potency of anti-D blood-grouping reagents. The candidate reference reagent was evaluated against a wide range of commercial anti-D blood-grouping reagents in an international collaborative study involving 20 laboratories in 13 countries (WHO/BS/04.2000, WHO/BS/04.2000 Add.1 and WHO/BS/04.2000 Rev.1).

Based on the data presented in the reports, reagent 99/836 was established by WHO as the first International Standard for minimum potency of anti-D blood-grouping reagents. An eightfold dilution of reconstituted 99/836 should define the minimum potency of high-protein anti-D blood grouping reagents and a threefold dilution of reconstituted 99/836 should define the minimum potency of low-protein anti-D blood-grouping reagents. Manufacturers should ensure that, in parallel haemagglutination titrations, their anti-D blood grouping reagents are at least as potent as 99/836 when reconstituted and diluted as described.

The Committee noted that stocks of 99/836 had been shared with CBER/FDA for distribution as the United States Minimum Potency Reference Reagent.

Factor V Leiden, first International Genetic Reference Panel

The factor V Leiden polymorphism results in a slower inactivation rate of activated factor V by activated protein C and it is associated with a 5–10-fold increase in the risk of venous thrombosis in heterozygotes and a 50–80-fold increase in homozygotes. The highest prevalence of the mutation has been found in European populations of Caucasian origin. In contrast the mutation appears to be rare among Chinese and absent in Africans and Japanese. As a consequence of the high incidence of the mutation, testing for factor V Leiden is one of the most frequent human genotyping tests performed in clinical laboratories. The frequency of testing is likely to increase as research explores links with proposed risk factors such as a previous history of thrombosis, use of oral contraceptives or hormone replacement therapy or proposed links between inherent genetic susceptibility for venous thrombosis and air travel. External quality assurance schemes have shown that errors in genotyping on factor V Leiden do occur. These errors can have a significant and long-lasting impact on the patient, particularly because genotyping tests are usually carried out only once on any one patient.

Most laboratories use blood or extracted DNA from samples from patients with the known polymorphism as their in-assay references. Currently there is no guaranteed supply of a stable and reliable reference material for this polymorphism and there is an urgent need for such materials. A panel of genomic DNA (gDNA) was extracted from immortalized cell lines produced by *Human herpesvirus 4* (Epstein–Barr virus) transformation of blood from donors who were known to carry the wild-type, homozygote and heterozygote genotypes for factor V Leiden, with a view to evaluating the suitability of these materials for inclusion in an internationally accepted Genetic Reference Panel for Factor V Leiden.

Forty-one laboratories from 16 countries took part in an international collaborative study to evaluate the suitability of the proposed panel of gDNA samples (WHO/BS/04.1997). The participants used 32 techniques with different underlying principles and within these techniques different individual protocols. To assess the consistency of the panel's performance, the participants were also requested to carry out the study over a period of 3 days and using different operators.

Preliminary accelerated degradation studies showed no detectable degradation in any of the samples by quantitative polymerase chain reaction.

Based on the results presented, the Committee endorsed the three human gDNA preparations 03/254 (FV wild type), 03/260 (FVL homozygote) and 03/248 (FVL heterozygote) as the First International Genetic Reference Panel for Factor V Leiden, Human gDNA. No unitage was assigned to the individual panel members. The materials provide a source of well characterized genotypes and are intended for use in the validation of new techniques or new test kits, or for validating existing techniques after a change in reagents, operator or equipment.

Blood coagulation factor XIII, plasma: first International Standard

Factor XIII (FXIII) is a transglutaminase, which covalently stabilizes a fibrin clot by cross- linking polymerized fibrin. FXIII is essential for maintaining haemostasis as it increases both the mechanical stability of the fibrin clot and resistance to plasmin degradation. Deficiencies in FXIII can lead to a severe bleeding diathesis and most patients with inherited FXIII deficiencies require lifelong supplementation therapy, primarily with FXIII concentrates. Currently there is only one manufacturer supplying plasma-derived FXIII concentrates, although a recombinant FXIII product is being developed by another manufacturer.

Measurement of FXIII levels is important in both patients' plasma and in concentrates. A study has shown that in some laboratories there is a high percentage of misclassification of FXIII deficiency, which clearly indicates the need for standardization.

FXIII is also a constituent of the fibrinogen component of fibrin sealants and the measurement of FXIII in fibrinogen components has been complicated because high fibrinogen concentrations give rise to variabilities in measurement.

Two main methodological approaches for the measurement of FXIII activity are in use. However, there is a clear need to standardize these methods to minimize the possibility of variations in procedure being contributory factors in discrepant observations. To date no reference preparations for measurement of FXIII have been established.

An international collaborative study, in which 23 laboratories in 10 countries were invited to calibrate a proposed International Standard for factor XIII (FXIII) plasma, was reported to the Committee (WHO/BS/04.1994 Rev.1). The participants included 14 academic

institutions, seven manufacturers and two national control laboratories but only 10 laboratories provided data. This study also investigated the relationships between measurements of FXIII in concentrates compared with those in plasma and between measurement of FXIII activity and FXIII antigen levels.

Estimates of FXIII potency for the candidate plasma standard Y (02/206) showed good agreement between laboratories. Furthermore there was negligible difference in potencies determined by the two methods currently in use.

Accelerated degradation studies showed that the proposed standard is very stable, with a predicted loss of activity per year of less than 0.06% at the recommended storage temperature of –20 °C. The stability/accelerated degradation study showed that this candidate plasma material is sufficiently stable to serve as a WHO standard.

The proposed International Standard was prepared from a pool of 17 healthy donors. All units of plasma were tested and found to be negative for hepatitis B surface antigen, antibodies to HIV-1 and HIV-2 and antibodies to hepatitis C.

The Committee endorsed the preparation Y (NIBSC code 02/206) as the first International Standard for Factor XIII plasma and assigned a value of 0.91 IU/ampoule for Factor XIII potency to it.

Immunoglobulin, intravenous: WHO reference reagents for anti-D content

The Committee was informed of the results of an international collaborative study to standardize and control haemagglutination tests for anti-D antibody in normal intravenous immunoglobulin (IVIG) (WHO/BS/04.2002). Twenty laboratories located in Australia, Europe and the USA participated in the collaborative study. A lyophilized IVIG preparation containing anti-D and a lyophilized negative control IVIG preparation were evaluated for their suitability as reference reagents.

The atypical presence of anti-D in IVIG has been linked to adverse reactions in recipients, including haemolysis, although at present there are no universal specifications or reference reagents to control the level of anti-D in IVIG or to standardize haemagglutination testing methodology. Even a relatively low level of contamination with anti-D can be significant with respect to the amount of IVIG infused into patients, especially if repeat doses are administered. However, detecting anti-D in IVIG using conventional indirect antiglobulin tests can be problematical as the high concentration of

immunoglobulin can neutralize the antiglobulin reagent resulting in false negatives, or result in rouleaux formation.

Using the specified direct microtitre-based haemagglutination method, there was reasonable consistency in titres for most preparations within most laboratories. However, a wide variation in haemagglutination titres between laboratories for three of the four samples was found. Correcting the titres of the samples relative to those of the proposed reference reagent reduced the interlaboratory variability and increased the frequency of the mode titres of three out of four samples. The indirect antiglobulin tests also showed wide variability between laboratories and were less sensitive than the direct method in four laboratories.

The results show that use of the reference reagents would help to overcome variability in haemagglutination tests between laboratories and also to ensure that such tests are sufficiently sensitive to detect anti-D in IVIG products. The EDQM and the CBER have recently taken the necessary steps to implement a test and a limit for anti-D in IVIG products. The establishment of standard preparations as WHO International Reference Reagents would facilitate global standardization of haemagglutination testing for anti-D in IVIG.

Based on the data in the report the Committee endorsed the Anti-D in IVIG Positive Control Reference Reagent, 02/228, and the Anti-D in IVIG Negative Control Reference Reagent, 02/226, as WHO Reference Reagents for use in standardizing haemagglutination testing methodology for anti-D in IVIG and ensuring that such tests are sufficiently sensitive to detect anti-D in IVIG. Eight hundred and fifty ampoules each of 02/228 and 02/226 are available for distribution as WHO Reference Reagents.

Cytokines, growth factors and endocrinological substances

Progress report on follow-up from the seventh WHO Consultation on cytokines, growth factors and endocrinological substances

The seventh WHO Informal consultation on Standards for Cytokines, Growth Factors and Endocrinological Substances was held at the NIBSC, Potters Bar, Herts., England, from 20–21 October 2003. Several issues relating to biological standardization and the establishment of new reference materials were discussed and subsequently presented at the fifty-fourth meeting of the Expert Committee on Biological Standardization, held from 17–21 November 2003. The

Committee took note from a status report (WHO/BS/04.2003) of the follow-up activities it had recommended.

The Committee reaffirmed that WHO should become more involved in the issue of standardized evaluations of unwanted immune responses to therapeutic proteins. They requested that a workshop on this topic be organized in conjunction with the next meeting of the WHO Working Group on Standards for Cytokines, Growth Factors and Endocrinological Substances. It was also reported to the Committee that data to evaluate a proposed method to harmonize the calculation and reporting of interferon neutralizing antibody tests may become available during 2005 and, if so, the outcome of this evaluation would be reported to the Committee.

The Committee also considered the outcome of an enquiry initiated by the Secretariat in response to a request to re-assign the potency of the Second WHO International Standard for human interferon alpha, lymphoblastoid N1, 95/568 to compensate for an error made during the calibration of a national standard. Consistent with the responses to the enquiry, and although aware of the difficulties that had arisen due to the miscalibration, the Committee decided, on principle, that the value assignment to the International Standard should not be changed. The Secretariat was requested to follow up the matter with the Member State concerned to determine what other course of action may be appropriate.

Diagnostic reagents

Global measurement standards for in vitro diagnostic devices: principles and priorities

The Committee was informed about a Consultation held in Geneva from June 7–8, 2004 and the conclusions drawn by the participants. The WHO Consultation had been held to discuss how the concepts of metrological traceability and measurement uncertainty could be applied to biological reference standards with values assigned in arbitrary units of biological activity. Data from WHO collaborative studies on selected blood coagulation and hormone protein standards and the Second International Standard for hepatitis B surface antigen were considered as models for discussion. The Committee adopted the report of the meeting because it was an important milestone in the resolution of key issues of principle that were subsequently incorporated into the revised recommendations on the preparation, characterization and establishment of international biological standards (see Annex 2).

The Committee was informed of the In Vitro Devices Directive (IVDD) of the European Union (98/79). This governs the use of diagnostics within the European Union from January 2004. Materials whose use affects patient treatment must be "CE marked" or be declared "higher order" references by the European Commission. It is assumed that all International Standards established by WHO would be categorized as higher order reference materials. The Committee was informed of the pressing need for a formal written declaration to this effect from the Commission covering all such preparations, without which the WHO International Laboratory at the NIBSC may be in breach of European law if it distributes new standards which might conceivably be used for diagnostic purposes. Standards in the distribution pipeline (i.e. those established before December 2003), would not be affected until December 2005. The standards established at the present meeting would be considered by the NIBSC as internationally certified and would be issued during 2005. Urgent discussions continue on a bilateral basis between the EU and WHO to try and resolve this issue.

WHO would give priority to diagnostic tests for infections such as HIV, hepatitis B and hepatitis C and the high-risk in vitro diagnostic devices and continue its collaboration with the other international standard-setting organizations and associations. The Committee recommended that the participants in this collaborative work should include experts from the manufacturers and from associations such as the International Society of Blood Transfusion.

Diagnostic tests for anti-hepatitis C virus: proposal for a reference standard and preliminary results

Information on the status of different assays for anti-hepatitis C virus (HCV) and the problems of establishing suitable reference materials was provided. A novel approach for WHO to antibody standardization was to evaluate the suitability of human antibody preparations processed to be monospecific for a target antigen. The initial results of a feasibility study of this approach for anti-HCV standardization were reported. One problem identified, even after immune absorption of the material, was that antibodies interfering with other antigens still remained in the preparation. The conditions of the immune absorption and the ammonium sulfate desorption may have contributed to some of the results obtained. The Committee recommended that further work be done to explore this approach and the continued expertise and contributions of manufacturers would be extremely valuable in collaboration with the work of the International Laboratory.

WHO Technical Report Series, No. 932, 2006

Annex 1
Guidelines for the production and quality control of candidate tetravalent dengue virus vaccines (live)

This document provides guidance to national regulatory authorities (NRAs) and vaccine manufacturers on the production and quality control of candidate live attenuated dengue virus vaccines by outlining the international regulatory expectations for product characterization. It should be read in conjunction with the WHO guidelines on nonclinical evaluation of vaccines (1), and the WHO guidelines on clinical evaluation of vaccines: regulatory expectations (2), to gain an understanding of the whole process of vaccine evaluation. Clinical evaluation of vaccines against dengue presents special challenges and WHO has developed specific guidance on clinical testing programmes (3), which should also be consulted. As candidate live attenuated dengue virus vaccines are still under development, the following text is presented in the form of guidelines rather than recommendations. Guidelines allow greater flexibility than recommendations with respect to expected future developments in the field. The document is thus provided for guidance to health administrators.

Introduction

In response to interest from many countries in the development of candidate live attenuated dengue virus vaccines, preliminary draft guidelines on production and quality control specifications for tetravalent dengue vaccine (live) were developed by a small drafting group established by WHO. These were presented to the WHO Expert Committee on Biological Standardization at its forty-eighth meeting in 1997 (*4*). The Committee advised that the field of dengue virus vaccine development needed to progress further before it would be appropriate for WHO to develop guidance on technical specifications for these vaccines. Since that time WHO had established the Task Force on Clinical Trials of Dengue Vaccines, and at the second meeting of this group, at Denver, Colorado, in November 2002, it was considered timely by the experts present for WHO to recommence the development of the production and quality control guidelines. The WHO Secretariat agreed and convened a small drafting group to review the previous draft and to advise on what changes should be made. The drafting group met in Geneva from 20–21 March 2003, reviewed the original document and, based on the conclusions from that meeting, subsequently developed a new draft. This draft was discussed in detail at a WHO consultation held in Philadelphia, USA, from 2–3 December 2003, and the current version of the document was prepared by the WHO Secretariat, taking into account the views expressed at that meeting (*5*) and the views of the fifty-fifth meeting of the Expert Committee on Biological Standardization.

The scope of this document covers candidate live attenuated tetravalent dengue virus vaccines. The aim of vaccination against dengue virus infection is to induce immunity against all four serotypes in one series of inoculations. The information available to the WHO Task Force on Clinical Trials for Dengue Vaccines in 2004 was that two tetravalent vaccine candidates had been generated by taking original patient isolates of each serotype and passaging these isolates in dog primary kidney cells to attenuate the viruses. Extensive testing has been done to define the attenuation phenotype for each of the vaccine candidates. Vaccine formulations are being developed based on the optimal degree of attenuation and immunogenicity. A third vaccine candidate had been generated from a molecular clone of dengue virus type 4. This virus contains a 30-nucleotide deletion in the 3′ noncoding region that attenuates the virus. To generate a tetravalent vaccine, chimeric vaccine candidates that contain the structural region of the other three dengue serotypes in a dengue virus type 4 backbone containing the 30-nucleotide deletion are being prepared

for clinical development. In addition the 30-nucleotide deletion has been introduced into the homologous region of dengue types 1, 2 and 3 to generate additional attenuated vaccine candidates. The fourth vaccine candidate was a chimeric vaccine made by expressing the dengue virus structural proteins, prM and E in a molecular clone of the yellow fever virus vaccine 17D backbone. A combination tetravalent vaccine was in development, which will have all four dengue serotypes, represented as chimeric dengue–yellow fever vaccines.

Clinical trial data (*6*, *7*) showed that vaccine candidates have induced human immune responses. Protection was also being measured in a human challenge study that was under way for at least one vaccine candidate. Additional data are required to define an acceptable level of immune response that correlates with protective immunity to candidate dengue vaccines. The information available to WHO suggested that the reactogenicity of the vaccine candidates being tested varied. To obtain maximum public health benefits, this vaccine was envisaged for use both in children and adults, thus it was important to establish and understand the safety profile and reactogenicity in all target age groups.

General considerations

An important consideration for the safety of any vaccine is the full passage history of the seed materials used for vaccine development. The purpose is to identify all substrates through which the seed materials had been passed to aid the development of appropriate programmes for testing for adventitious agents. The early passage history for the candidate dengue vaccines varies, but may include monkey kidney cells, or mosquito intermediates, or mouse brain or dog primary kidney cells, or a combination of these substrates. It will be essential to show that the virus seeds are free of adventitious agents relevant to the animal species used and from the substrates used in the derivation of the seeds.

A risk assessment for transmissible spongiform encephalopathies (TSE) would need to be included for the seed materials. The revised WHO Guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products (*8*) provide guidance on risk assessments for master and working seeds and should be consulted.

All of the vaccine candidates are claimed to be attenuated. However, for some vaccine candidates, this claim may be based on limited

clinical experience in humans as laboratory markers of attenuation are not well-defined. This is an area where further work is urgently needed to help assess consistency of production, especially when new viral seeds are produced. Potential laboratory markers include the sequence information on the seed viruses; viraemia levels in monkeys; and ability to replicate in or be transmitted by vector mosquitoes. In any laboratory test, the aim would be to show that a new seed material was similar to the previous seed and that each could be distinguished from the parent virus. Multiple passage of virus seed materials under defined conditions may be helpful to generate validation data on the chosen method. Studies on consistency of production would need to take into account the variability inherent in RNA virus replication and assess the presence of minority populations, as revealed for example by the occurrence of mixed plaque phenotypes or mixed base signals in sequencing studies. If minority populations are detected it will be necessary to assess their biological importance, for example, by carefully comparing the level of heterogeneity (e.g. plaque size) between the master or working seed and higher passage levels (e.g. clinical trial material).

The need for a neurovirulence test should be based on evidence (*2*) either that the natural infection is neurotropic or that selection for neurotropism could have occurred during the passage history of the vaccine candidates. For example, this may occur if the attenuation process involved passage through central nervous system (CNS) tissue. Furthermore, if a neurovirulence test is indicated, any test specified should be able to reliably distinguish between acceptable and unacceptable preparations.

Because dengue viruses are not regarded as encephalitic, a neurovirulence test for each batch is not justified, and there is no need to test each working seed. However, it would be prudent to test at least the master seed to show that the derivation process for the seed did not lead to a change in the inherently attenuated neurovirulence phenotype of dengue viruses. For chimeric dengue candidate vaccines where one component of the chimera is derived from a virus with neurovirulence potential, such as dengue–yellow fever constructs, then more extensive neurovirulence testing may be required. Experience to date with dengue neurovirulence testing is based on using either the methodology described for live attenuated poliovirus (*9*) or for yellow fever vaccine (*10*). It is proposed that, because both yellow fever and dengue viruses are *Flaviviruses*, the specifications for the yellow fever test be applied to dengue virus vaccines in the future. Thus a neurovirulence test at the level of the master seed is included.

Two candidate vaccines are being developed in Vero cells. These are among the first examples of live-attenuated injectable vaccines for human use being developed in Vero cells. The existing WHO guidance on residual levels of cellular DNA (*11*, *12*) is incorporated into this draft. It is recognized that this specification will require that either a virus purification step and/or a DNA removal step be included in the production process.

For vaccines being developed in fetal rhesus lung diploid cells, existing WHO recommendations are applicable (*11*).

Although continuous cell lines or diploid cells are generally the preferred cell substrates for vaccine production, at least one candidate vaccine was produced in dog primary kidney cells. These cells are passaged a limited number of times and banked prior to use. Thus it is possible to conduct extensive characterization of adventitious agents in the cells. The principles of extensive testing of source animals plus extensive testing of each batch of cells, using as an example the guidance established for primary hamster cells for production of live attenuated Japanese encephalitis vaccine (*13*), have been applied in this document. Also the conclusions of a WHO Task Force meeting on cell substrates, which considered the issue of dog primary kidney cells (*14*) have been taken into account. Thus, for example, testing for canine retroviruses is included.

The infectivity of each serotype in a tetravalent mixture should be established and the plaque or focus-forming assay is specified for determinations of infectivity. Candidate titration standards do not exist at present and WHO should consider developing such reagents and their subsequent characterization by international collaborative study.

The thermal stability of the final tetravalent freeze-dried product should be determined in an appropriate stability study. This study should determine the thermal stability of each serotype in the tetravalent mixture. In addition to the stability of the freeze-dried product, the stability of the liquid vaccine after reconstitution should also be studied. Stability testing of intermediates, such as monovalent virus harvests prior to formulation as final tetravalent vaccine, is required in some countries. WHO is developing further guidance on this issue.

Based on the results of the stability testing programme, an accelerated degradation test should be conducted on each new batch of vaccine. This is to show the consistency of manufacture of the final stabilized formulation. For consistency with the testing done on other

vaccines the accelerated degradation test should be done at 37 °C for 1 week. A specification for the maximum allowable loss of titre during this period should be confirmed on the basis of experience yet to be accumulated.

Considerations for vectored vaccines for human use have been reviewed by WHO (*15*) and the general principles identified should be applied to the vaccine candidates derived by molecular methods.

Nonclinical testing of candidate dengue vaccines should follow conventional procedures (*1*), but in addition should address the issue of enhancement of antibody-mediated disease. There are no models that can be recommended at present, so each testing programme will need to be developed on a case-by-case basis. In addition, appropriate follow-up of vaccinees participating in human clinical trials for 3–5 years is recommended (3).

Theoretical concerns have been raised about adverse ecological events that may arise from recombination between a live attenuated dengue virus vaccine and a wild-type flavivirus (*16*). Scientific considerations show however that the likelihood of recombination between a wild-type flavivirus and a vaccine flavivirus is much less than that of recombination between two wild-type flavivirus. There is no evidence for generation of problematic recombinant flaviviruses (*17*). Dual infection laboratory studies between vaccine and wild-type strains are not recommended because the predictive value of such studies would be low (*18*).

Part A. Control of production

A.1 Definitions

A.1.1 *International name and proper name*

Although dengue vaccines are not yet licensed, 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 "Live attenuated tetravalent dengue virus vaccine" or "Live attenuated tetravalent dengue–yellow fever virus chimeric vaccine" or "Live attenuated tetravalent dengue–dengue 4 virus chimeric vaccine". 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 candidate live attenuated dengue virus vaccine should be a sterile, aqueous suspension of the four serotypes of dengue vaccine strains, or viral vectors that express the four dengue serotypes, and which have been grown individually in mammalian cells. At least three types of dengue vaccine are in development. These are as follows:

- Live attenuated tetravalent dengue virus vaccine is a preparation of combined live attenuated dengue-1, dengue-2, dengue-3 and dengue-4 viruses grown in a suitable cell culture.
- Live attenuated tetravalent dengue–yellow fever virus chimeric vaccine is a preparation of combined live attenuated chimeric viruses, based on the live attenuated yellow fever virus vaccine and each expressing dengue-1, dengue-2, dengue-3 or dengue-4 virus envelopes.
- Live attenuated tetravalent dengue–dengue 4 virus chimeric vaccine is a preparation of combined live attenuated chimeric viruses, based on the live attenuated dengue-4 vector and each expressing dengue-1, dengue-2, dengue-3 or dengue-4 virus envelopes.

The preparation should satisfy all of the specifications given below.

Live tetravalent dengue vaccine is blended with an appropriate stabilizer and may be freeze-dried.

A.1.3 *International reference materials*

No international reference materials are available at present, although candidate antiserum preparations to calibrate the neutralizing antibody response in vaccinees are under evaluation in a WHO collaborative study[a].

A.1.4 *Terminology*

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

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

Cell seed
A quantity of well-characterized cells of human or animal origin stored frozen in liquid nitrogen in aliquots of uniform composition

[a] The 1st WHO Reference Regent for anti-dengue antibodies was established in 2005 by the 56th meeting of the Expert Committee on Biological Standardization. This material was assigned a unitage of 100 units per serotyse.

derived from a single tissue or cell, one or more of which would be used for the production of a master cell bank.

Cell substrates
A number of cell cultures derived from the same pool of cells, processed and prepared together.

Filling lot
A collection of sealed final containers of finished candidate vaccine that are homogeneous with respect to the risk of contamination during filling and freeze-drying. All the final containers must, therefore, have been filled from one vessel of final bulk in one working session and freeze-dried under standardized conditions in a common chamber.

Final bulk
The homogeneous finished tetravalent virus suspension prepared from one or more clarified virus pools in the vessel from which the final containers are filled.

Focus-forming unit (FFU)
The smallest quantity of virus suspension that can be defeated using dengue-specific antisera in monolayer cell cultures.

Master cell bank
A quantity of fully characterized cells of human, animal or other origin stored frozen at –70 °C or below in aliquots of uniform composition, one or more of which would be used for the production of a manufacturer's working cell bank.

Master seed lot
A quantity of virus derived from an original isolate, processed at the same time to assure a uniform composition and having been characterized to the extent necessary to support developing the working seed lot. The characterized master seed lot is used for the preparation of working seed lots.

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

Single harvests
A quantity of virus suspension derived from the batch of cell substrate that was inoculated with the same working seed lot and processed together in a single production run.

Working cell bank (WCB)
A quantity of cells derived from one or more ampoules of the master cell bank and of uniform composition, stored frozen at –70 °C or below in aliquots, one or more of which would be used for production purposes.

In normal practice a master cell bank is expanded by serial subculture up to a passage number (or population doubling, as appropriate) selected by the manufacturer and approved by the national authority. The cells are combined into one pool distributed into ampoules and preserved cryogenically to form the WCB.

Working seed lot
A quantity of virus of uniform composition, fully characterized, derived from a master seed lot. The working seed lot is used for the production of candidate vaccine lots.

Virus pool
A homogenous pool of single harvests collected into a single vessel before clarification.

A.2 Viruses for use in candidate vaccine production

A.2.1 *Virus strains*

The strains of dengue-1, dengue-2, dengue-3 and dengue-4 viruses used in the production of candidate tetravalent dengue vaccine should be identified by historical records, which will include information on the origin of each strain; method of attenuation; whether the strains have been biologically cloned prior to generation of the master seed; genetic sequence information; and the passage level at which attenuation for humans was demonstrated by clinical trials. Clinical signs, viraemia, and the immune response after human immunization with each dengue virus serotype must be determined to facilitate development of acceptable criteria for attenuation and immunogenicity of the vaccine viruses.

A.2.2 *Approval*

The four vaccine strains of dengue virus used in the production of candidate vaccine should have been shown to be safe by appropriate laboratory tests (see section A.4 of these guidelines) as well as by tests in susceptible humans. Only strains approved by the national regulatory authority should be used.

A.2.3 *Establishment of immunizing dose*

The immunizing dose, initially in PFU or FFU, of each serotype in the tetravalent vaccine that induces seroconversion when susceptible individuals are immunized with the tetravalent vaccine, should be established in a dose–response study. Any potential interference or potentiation between the serotypes in the plaque- or focus-forming assay should be evaluated prior to establishing this value. When international reference standards become available, the immunizing dose should be expressed relative to the standard. Expression of doses as a relative potency is encouraged because experience shows that this reduces variation between laboratories. The immunizing dose should also serve as a basis for establishing parameters for stability and expiry date.

A.3 General manufacturing requirements

The principles of good manufacturing practices for pharmaceutical and biological products, as appropriate to the different stages of vaccine development, should be applied by establishments manufacturing candidate tetravalent dengue vaccine (*22*), with the addition of the following:

Separate manufacturing areas for each of the four serotypes as well as tetravalent vaccine formulation are required. 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 biosafety level BSL 2.

A.4 Production control

A.4.1 *Control of source materials*

A.4.1.1 *Cell cultures for virus production*

A.4.1.1.1 Conformity with WHO requirements

Dengue viruses used in the production of tetravalent dengue vaccine should be propagated in cell substrate in conformity with the WHO requirements for use of animal cells as in vitro substrate for the production of biologicals (*11*, *12*) and approved by the national regulatory authority. All information on the source and method of preparation of the cell culture system used should be made available to the national regulatory authority (*11*).

A.4.1.1.2 Types of cell culture

Dengue vaccine candidates have been produced in fetal rhesus lung diploid cells, in continuous cell lines and in dog primary kidney cells. For fetal rhesus lung diploid and continuous cells, sections A.4.1.1.3 and A.4.1.1.4 should apply; for dog primary kidney cells, section A.4.1.1.5 should apply to the source materials. Section A.4.1.1.6 applies to all types of cell culture.

A.4.1.1.3 Master cell bank and working cell bank

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 national regulatory authority. The maximum number of passages (or population doublings) allowable between the cell seed and the WCB should be established by the national regulatory authority. Additional tests for Vero cells include:

— propagation of the MCB or WCB cells to or beyond the maximum in vitro age; and
— examination for the presence of retroviruses and tumorigenicity in an animal test system (*11*).

WHO has established a cell bank of Vero cells characterized in accordance with the requirements in the report of the WHO Expert Committee on Biological Standardization (*11*), which is available to manufacturers as a well-characterized starting material (*12*) for preparation of their own master and working cell seeds on application to the Coordinator, Quality Assurance and Safety of Biologicals, WHO, Geneva, Switzerland.

A.4.1.1.4 Identity test

The master cell bank should be characterized according to the *Requirements for animal cells lines used as substrates for the production* of *biologicals* (*11*), as appropriate to continuous cells or fetal rhesus diploid cells.

The WCB should be identified by means, inter alia, of biochemical (e.g. isoenzyme analysis), immunological, and cytogenetic marker tests, approved by the national regulatory authority.

A.4.1.1.5 Sources of dog kidney cells

If cultures of dog kidney cells are used for the propagation of dengue vaccine viruses, dogs less than 2 months old may be used as the source of kidneys for cell culture. Only dog stock approved by the national

regulatory authority should be used as the source of tissue and should be derived from a closed, healthy colony. A closed colony is a group of animals sharing a common environment and having their own caretakers who have no contact with other animal colonies. The animals are tested according to a defined programme to ensure freedom from specified pathogens and their antibodies. When new animals are introduced into the colony for breeding purposes, they should be maintained in quarantine in vermin-proof quarters for a minimum of 2 months and shown to be free from these specified pathogens. The parents of animals to be used as a source of tissue should also be maintained in vermin-proof quarters. Neither parent dogs nor their progeny should previously have been used for experimental purposes, especially those involving infectious agents. The colony should be monitored for zoonotic viruses and markers of contamination at regular intervals.

At the time the colony is established, all animals should be tested to determine freedom from antibodies to the following pathogens: rabies, canine parvovirus, canine distempervirus, canine adenovirus 1, canine adenovirus 2, parainfluenzavirus 3, Sendai virus, SV-5 virus, reovirus types 1, 2, 3, *Mycobacterium tuberculosis*, infectious canine hepatitis and leptospirosis. Following this initial screening, a monitoring programme should be implemented to ensure that the colony remains free of the specified pathogens.

In some countries, the whole group of animals is bled on the establishment of the colony, and thereafter 5% of the animals should be bled each month. The screening programme should test all of the animals over a defined period of time, as agreed with the national regulatory authority. The serum samples should be screened to establish freedom from antibodies to the pathogens above.

Consideration should also be given to testing the colony for hepatitis E virus, Japanese encephalitis, canine circovirus, canine coronavirus, canine herpesviruses and bordetella bronchiseptica.

The colony should be tested for retroviruses using a sensitive polymerase chain reaction (PCR)-based reverse transcriptase (Rtase) assay. The results of such assays may need to be interpreted with caution because Rtase activity is not unique to retroviruses and may derive from other sources, such as retrovirus-like elements that do not encode a complete genome (*19*). If a positive result is obtained in this screening, it is then important to determine whether replication-competent retroviruses are present. It should be noted that dogs have many classes of defective endogenous retroviruses but, as yet, no definitely characterized exogenous retrovirus.

Any animal that becomes ill or dies should be investigated to determine if the cause of illness or death may be of infectious origin. Similarly, ill health of animals within the colony should be investigated to determine if the cause is infectious in origin. If an infectious agent is detected in the colony, specific steps must be put in place to ensure that the agent is excluded from the candidate vaccine.

The dog kidney cell cultures currently in use are generated from harvested tissue that is passaged up to three times and then stored as a working cell bank. This enables these cells to be characterized in more detail than if they were used without intervening passage. Nevertheless, these cells are still considered primary cells. Given the inherent variability of primary cell cultures and to ensure consistency of manufacturing, it is recommended that the characterization of any new dog primary kidney cell bank (third passage cells) include tests comparing cells currently in use with newly harvested and passaged cells.

A.4.1.1.6 Cell culture medium

Serum used for the propagation of cells should be tested to demonstrate freedom from bacteria, fungi and mycoplasmas, according to the requirements given in Part A, sections 5.2 and 5.3 of the revised *Requirements for biological substances*, No. 6 (*20*), and from infectious viruses. Suitable tests for detecting viruses in bovine serum are given in Appendix 1 of the *Recommendations for production and control of poliomyelitis vaccine (oral)* (*9*).

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

As an additional monitor of quality, sera may be examined for freedom from phage and endotoxin.

Irradiation may be used to inactivate potential contaminant viruses.

The acceptability of the sources(s) of any components of bovine, sheep or goat origin used in culture media should be approved by the national regulatory authority. These components should comply with current guidelines in relation to animal transmissible spongiform encephalopathies (*8*).

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) (*21*), as well as current guidelines in relation to human transmissible encephalopathies (*8*).

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.

Penicillin and other beta-lactams should not be used at any stage of the manufacture. Other antibiotics may be used at any stage in the manufacture provided that the quantity present in the final product is acceptable to the national regulatory authority. Nontoxic pH indicators may be added, e.g. phenol red at a concentration of 0.002%. Only substances that have been approved by the national regulatory authority may be added.

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

The source(s) of trypsin of bovine origin, if used, should be approved by the national regulatory authority. Bovine trypsin, if used, should comply with current guidelines in relation to animal transmissible spongiform encephalopathies (*8*).

A.4.1.2 *Virus seeds*

A.4.1.2.1 **Virus strains**

Virus strains of dengue viruses used for master and working seeds to produce vaccine candidates should comply with the specifications of section A.2. Strains derived by molecular methods may be used, provided that guidance on vectored vaccines is taken into account (*15*). Viruses may be passed in continuous, diploid, and/or primary cell lines. The candidate vaccine strains should be approved by the national regulatory authority.

If molecularly derived strains are used, and because this is a live attenuated vaccine, the candidate vaccine should be considered a genetically modified organism (GMO) and should comply with the regulations of the producing and recipient countries regarding GMOs. An environmental risk assessment should be undertaken.

A.4.1.2.2 **Molecularly derived strains**

The genomes of the viruses in these candidate vaccines may be genetically altered and may consist of intentionally introduced mutations or

deletions, genetic elements from one or more of the dengue virus strains, or genetic elements of related flaviviruses. The primary virus seed is made from the transfection of in vitro-generated viral RNA transcripts that are synthesized from a well-characterized, full-length cDNA clone template into an appropriate cell substrate.

The sequence of any cDNA clone used to generate vaccine virus stocks must be determined prior to transfection of viral RNA into the defined host cell substrate. Virus stocks to be used as working virus seeds, derived from passaging of the primary virus stock, should also be sequenced.

Viral vaccine seeds rederived by cDNA cloning to reduce the risk of TSE contamination are considered as new vaccine candidates and appropriate bridging studies, including clinical studies, should be performed to demonstrate similarity to the starting virus seed.

A.4.1.2.3 Virus seed lot system

The production of vaccine should be based on the master and working seed lot system. Seed lots should be prepared in the same type of cells as those used for production of final vaccine.

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

A.4.1.2.3.1 Tests on virus master seeds

1. Identity

Each master seed lot should be identified as dengue virus type-1, 2, 3 or 4 by immunological assay or by sequencing.

2. Genotype/phenotype characterization

Each seed should be characterized by full-length sequence and by other relevant laboratory and animal tests. Genotype and phenotype stability of the seeds upon passage should be measured using relevant assays to ensure uniformity of vaccine lots. It should be noted that full-length sequencing may not identify minority populations of variants that may be present in candidate vaccines.

3. Freedom from bacteria, fungi and mycoplasmas

Each master seed lot should be shown to be free from bacterial, mycotic and mycoplasmal contamination by appropriate tests as specified in Part A, sections 5.2 and 5.3, of the revised *Requirements*

for biological substances, No. 6 (General Requirements for the Sterility of Biological Substances, 1995) (*20*).

4. Tests for adventitious viruses

Each master seed lot should be tested in cell cultures for adventitious viruses relevant to the passage history of the seed virus. Neutralization of dengue virus is necessary for many tests because the virus is cytopathogenic. Antisera used for this purpose should be shown to be free from antibodies that may neutralize the specific adventitious virus being tested for. The cells inoculated should be observed microscopically for cytopathic changes. At the end of the observation period, the cells should be tested for haemadsorbing viruses.

Each master or working seed lot should also be tested in animals that include guinea-pigs, mice and embryonated chicken eggs, as appropriate.

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

5. Testing in non-human primates

Neurotropism test

To provide some level of assurance that a candidate vaccine will not be unusually neurovirulent, each master seed lot of each serotype should be tested for neurovirulence in monkeys by inoculation of *Macaca mulatta* (rhesus), Cynomolgus or other susceptible species of monkey. Tests should follow the WHO Requirements for yellow fever vaccine (*10*). Groups of at least 10 monkeys, demonstrated to be non-immune to dengue viruses and yellow fever virus immediately prior to inoculation of the seed virus, should be inoculated intracerebrally into the frontal lobe. A control group of 10 monkeys, also demonstrated to be non-immune to dengue viruses and yellow fever virus immediately prior to inoculation of the seed virus should receive yellow fever vaccine strain 17D as the control group.

The neutralizing antibody test should be used to assess immune status to dengue virus and yellow fever virus.

All monkeys should be observed for a period of 30 days for signs of encephalitis. Clinical scores, and the severity of histological lesions of the central nervous system, of the test group should not exceed scores of the control (yellow fever vaccine) group.

Viscerotropism test

For some vaccine candidates, evaluation of the master seed virus of each serotype for viscerotropism by assay of viraemia may be con-

sidered as an additional characterizing parameter. The method and specifications for yellow fever virus vaccine (*10*) should be followed.

6. Virus titration for infectivity

Each master seed lot should be assayed for infectivity in a sensitive assay in cell cultures.

- A plaque assay may be used in Vero or other sensitive cells. Titre should be determined by counting the number of visible plaques developed, and results recorded as PFU/ml.
- An immunofocus assay may also be used to measure virus titre. The assay is based on the visualization of infected areas of a cell monolayer by probing with dengue serotype-specific monoclonal antibodies. Results should be recorded as FFU/ml.
- A tissue culture infectious dose assay may also be used to determine virus titre. Results should be recorded as cell-culture infectious dose $(CCID)_{50}$/ml.

A.4.1.2.3.2 Tests on virus working seeds

The virus working seed lot is used for the production of vaccine batches and is prepared from a qualified virus master seed lot approved by the national regulatory authority. The working seed lot should be limited to a specified number of passages in cell culture beyond the master seed lot.

1. Identity

Each working seed lot should be identified as dengue virus type-1, 2, 3 or 4 by immunological assay or by sequencing.

2. Genotype/phenotype characterization

Each working seed should be characterized by full sequence and by other relevant laboratory and animal tests. Genotype and phenotype stability of the seeds upon passage should be measured using relevant assays to ensure uniformity of vaccine lots. Samples from vaccine lots that have been used for human clinical trials should be available in sufficient amounts to serve as future reference materials.

3. Freedom from bacteria, fungi and mycoplasmas

Each working seed lot should be shown to be free from bacterial, mycotic and mycoplasmal contamination by appropriate tests as specified in Part A, sections 5.2 and 5.3, of the revised *Requirements for biological substances* No. 6 (General Requirements for the Sterility of Biological Substances 1995) (*20*).

The absence of interference by the test articles in the sterility tests should be demonstrated.

4. Tests for adventitious viruses

Each working seed lot should be tested in cell cultures for adventitious viruses appropriate to the passage history of the seed virus. Neutralization of dengue virus is necessary for many tests because the virus is cytopathogenic. Antisera used for this purpose should be free from antibodies that may neutralize the adventitious virus being tested for. 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 PCR amplification techniques.

5. Virus titration for infectivity

Each working seed lot should be assayed for infectivity in a sensitive assay in cell cultures.

- A plaque assay may be used in Vero or other sensitive cells. Titre should be determined by counting the number of visible plaques developed, and results recorded as PFU/ml.
- An immunofocus assay may also be used to measure virus titre. The assay is based on the visualization of infected areas of a cell monolayer by probing with dengue serotype-specific monoclonal antibodies. Results should be recorded as FFU/ml.
- A tissue culture infectious dose assay may also be used to determine virus titre. Results should be recorded as $CCID_{50}$/ml.

If international reference standards become available, immunizing doses should be expressed as relative potencies rather than $CCID_{50}$ because experience shows that this reduces variability between assays.

A.4.2 *Control of vaccine production*

A.4.2.1 *Control cell cultures*

From the cell suspension used to prepare cell cultures for growing attenuated dengue viruses, an amount of processed cell suspension equivalent to at least 5% or 500 ml of cell suspension, whichever is greater, should be used to prepare control cultures of uninfected cells. These control cultures should be observed microscopically for changes attributable to the presence of adventitious agents for at least 14 days after the day of inoculation of the production cultures, or until the time of final virus harvest, if this is later. At the end of the observation period, fluids collected from the control culture should be tested for the presence of adventitious agents as described below (A.4.2.1.2). Samples that are not tested immediately should be stored at –60 °C or lower.

In some countries, samples of fluid from each control vessel are collected at the time of harvest. If several virus harvests are made from the same cell culture lot, the control fluid taken at each harvest is frozen and stored at or below –60 °C until the last virus harvest from that tissue culture lot has been taken. The control fluids are then pooled and submitted for testing.

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

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

A.4.2.1.1 Test for haemadsorbing viruses

At the end of the observation period, cells comprising no less than 25% of the control cells should be tested for the presence of haemadsorbing viruses, using guinea-pig red blood cells. If the red blood cells have been stored, the duration of storage should not have exceeded 7 days, and the storage temperature should have been in the range of 2–8 °C.

In some countries, the national regulatory authority requires that additional tests for haemadsorbing viruses 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 an 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 nonspecific accidental reasons by the end of the test period.

A.4.2.1.2 Tests for cytopathic, adventitious agents in control cell fluids

Control cell fluids collected at the time of harvest should be used for testing. A 10-ml sample of the pool should be tested in the same substrate, but not the same batch as that used for virus growth, and an additional 10-ml sample of each pool should be tested in both human and 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 in 4. The area of the cells should be at least 3 cm^2 per ml of pooled fluid. At least one bottle of the cell cultures 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 national regulatory authorities require that, at the end of this observation period, a subculture is made in the same culture system and observed for at least 7 days. Furthermore, some national control authorities 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 nonspecific accidental reasons by the end of the test period.

A.4.2.1.3 Additional tests if dog cell cultures are used

If dog cell cultures are used, a sample of fluids pooled from the control cultures should be tested for retroviruses, by a method approved by the national regulatory authority.

A test for retroviruses using a sensitive PCR-based reverse transcriptase (Rtase) assay may be used. The results of such assays need to be interpreted with caution because Rtase activity is not unique to retroviruses and may derive from other sources, such as retrovirus-like elements that do not encode a complete genome (*19*). Nucleic acid amplification tests for retrovirus may also be used.

A.4.3 *Production and harvest of monovalent vaccine virus*

A.4.3.1 *Cells used for vaccine production*

On the day of inoculation with the working seed virus, each cell culture and/or cell culture control should be examined for degeneration caused by infectious agents. If such examination shows evidence of the presence in a cell culture of any adventitious agent, the whole group of cultures concerned should not be used for vaccine production.

If animal serum is used in the growth medium for the cell cultures, the serum should be removed from the cell culture either before or after inoculation of working seed virus. Prior to beginning virus harvests, the cell cultures should be rinsed and the growth medium 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 national regulatory authority.

A.4.3.2 *Virus inoculation*

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

The optimal multiplicity of infection, temperature range and duration of incubation will depend on the vaccine strain and production method, but specifications should be given by each manufacturer. For multiplicity of infection, the specified range should not be greater than 10-fold and for temperature, the specified range should not be greater than +/– 1.5 °C.

A.4.3.3 *Monovalent virus harvest pools*

Vaccine virus is harvested within a defined time-period post-inoculation. A monovalent harvest may be the result of one or more single harvests. If several single harvests are taken, each single harvest should be stabilized and stored at 2–8 °C until pooling. No antibiotics should be added at the time of harvesting or at any later stage of manufacture. Samples of monovalent virus harvest pools should be taken for testing and stored at a temperature of –60 °C or below.

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 final bulk for freeze-drying.

The national regulatory authority may require the further purification of harvests derived from continuous cell lines to remove cellular DNA, and/or the use of DNAase treatment to reduce the size of DNA fragments. If the harvests are derived from human diploid or primary cell cultures, further purification is not required.

A.4.3.4 *Tests on monovalent virus harvest pools*

1. Identity

Each monovalent virus harvest pool should be identified as the appropriate dengue virus serotype by immunological assay or by sequencing (see section A.6.1).

2. Freedom from bacteria, fungi and mycoplasmas

Each monovalent virus harvest pool should be shown to be free from bacterial, mycotic and mycoplasmal contamination by appropriate tests as specified in Part A, sections 5.2 and 5.3, of the revised *Requirements for biological substances* No. 6 (General Requirements for the Sterility of Biological Substances, 1995) (*20*).

3. Tests for adventitious viruses

Each monovalent virus harvest pool should be tested in cell cultures for adventitious viruses appropriate to the passage history of the seed virus. Neutralization of dengue virus is necessary for many tests because the virus is cytopathogenic. Antisera used for this purpose should be free from antibodies that may neutralize the adventitious virus being tested for. 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 PCR amplification techniques.

4. Virus titration for infectivity

Each monovalent virus harvest pool should be assayed for infectivity in a sensitive assay in cell cultures.

- A plaque assay may be conducted using Vero or other sensitive cells. Titre should be determined by counting the number of visible plaques developed, and results recorded as PFU/ml.
- An immunofocus assay may also be used to measure virus titre. The assay is based on the visualization of infected areas of a cell monolayer by probing with dengue serotype-specific monoclonal antibodies. Results should be recorded as FFU/mL.
- A tissue culture infectious dose assay may also be used to determine virus titre. Results should be recorded as $CCID_{50}$/ml.

5. Tests for cellular DNA

For viruses grown in continuous cells the monovalent harvest pool should be tested for residual cellular DNA. The removal process, at production scale, should be shown to reduce consistently the level of cellular DNA to less than 10 ng per human dose. This test may be omitted, with the agreement of the national regulatory authority, if the manufacturing process is validated to achieve this specification.

6. Test for consistency of virus characteristics

The dengue virus in the monovalent harvest pool should be tested to compare it with the working seed virus, or suitable comparator, to

ensure that the vaccine virus has not undergone critical changes during its multiplication in the production culture system. The results of these tests for successive batches of vaccine should enable an assessment to be made of the consistency of vaccine production.

Attenuation assays for dengue viruses include reduced titre in tissue culture, small plaque phenotype, temperature sensitivity, and decrease in pathogenesis in an animal model. Other assays may be used if validated for this purpose.

Assays for the attenuation of dengue–yellow fever virus chimeric vaccines include tests in suckling and adult mice. Intracerebral inoculation of suckling mice with 10-fold dilutions of vaccine and yellow fever 17D is followed by the determination of the mortality ratio and survival time. Intracerebral inoculation of adult mice with undiluted virus seed or vaccine as compared with yellow fever 17D strain is also performed.

7. Storage

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

A.4.3.5 *Final tetravalent bulk lot*

The final tetravalent vaccine lot should be prepared from bulk lots 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 such a manner as to avoid contamination of the product.

In preparing the final bulk, any substance, such as diluent or stabilizer, that is added to the product should have been shown to the satisfaction of the national regulatory authority not to impair the safety and efficacy of the vaccine in the concentration used.

A.4.3.5.1 **Tests on the final tetravalent bulk lot**

Residual animal serum protein. A sample of the final bulk should be tested to verify that the level of serum is less than 50 ng per human dose. Alternatively the test may be performed on the clarified monovalent bulk.

Sterility. Each final bulk suspension should be tested for bacterial and mycotic sterility according to Part A, sections 5.2 and 5.3 of the Requirements for Biological Substances No. 6 (General Requirements for Sterility of Biological Substances) (*20*), or by a method approved by the national regulatory authority.

A.4.3.5.2 Storage

Until it is distributed into containers and lyophilized, the final bulk suspension should be stored under conditions shown by the manufacturer to retain the desired immunogenic activity.

A.5 Filling and containers

The requirements concerning good manufacturing practices for biological products (*22*) appropriate to a developmental vaccine should apply.

Care should be taken to ensure that the materials of which the container and, if applicable, the closure is made do not adversely affect the virus content of the vaccine under the recommended conditions of storage.

A final filtration could be included during the filling operations.

The manufacturer should provide the national regulatory authority with adequate data to prove the stability of the product under appropriate conditions of storage and shipping.

A.6 Control tests on final product

The following tests should be carried out on the final product.

1. Identity

Each tetravalent vaccine virus lot should be identified as dengue virus type-1, 2, 3 or 4 by immunological assay or by sequencing.

2. Virus titration for infectivity

Each tetravalent vaccine virus lot should be assayed for infectivity in a sensitive assay in cell cultures in which interference or potentiation between serotypes does not occur. The titre of each individual serotype should be determined.

- A plaque assay may be used in Vero or other sensitive cells. Titre should be determined by counting the number of visible plaques developed, and results recorded as PFU/ml.
- An immunofocus assay may also be used to measure virus titre. The assay is based on the visualization of infected areas of a cell monolayer by probing with dengue serotype-specific monoclonal antibodies. Results should be recorded as FFU/ml.
- A tissue culture infectious dose assay may also be used to determine virus titre. Results should be recorded as $CCID_{50}$/ml.

3. Accelerated degradation tests

Three containers of the final freeze-dried tetravalent vaccine should be incubated at 37 °C for 7 days. The geometric mean infectious

virus titre of the containers that have been exposed should not have decreased by more than a specified amount during the period of exposure. Titration of non-exposed and exposed vials should be done in parallel and results expressed in terms of PFU or $CCID_{50}$ or FFU per human dose. A reference reagent of each of the four dengue serotype viruses should be included in each assay to validate the assay.

The maximum allowable loss of titre during the accelerated degradation test should be confirmed on the basis of experience yet to be accumulated.

4. Sterility test

Reconstituted vaccine should be tested for bacterial and mycotic sterility according to the requirements in Part A, section 5.2 of the Requirements for biological substances No. 6 (Requirements for the sterility of biological substances) (*20*), by acceptable methods approved by the national regulatory authority.

5. General safety tests

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

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

6. Residual moisture

The residual moisture in a representative sample of each freeze-dried lot should be determined by a method approved by the national regulatory authority and an appropriate limit should be set by them.

Generally, moisture levels of 2% and less are considered satisfactory although some candidate vaccines formulations may be satisfactory at levels of up to 4%.

7. Inspection of final containers

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

A.7 Records

The requirements of Good manufacturing practices for biological products (*22*) pp. 27–28, should apply, as appropriate for 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 human clinical trials may serve as reference materials in the future and a sufficient number of vials should be reserved, and appropriately stored, for that purpose.

A.9 Labelling

The requirements of Good Manufacturing Practices for Biological Products (*20*) pp. 26–27, appropriate for a candidate vaccine should apply, with the addition of the following:

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

— a statement that the candidate vaccine fulfils Part A of these Requirements;
— a statement of the nature of the preparation, specifying the designation of the strains of dengue viruses contained in the live attenuated tetravalent vaccine, the minimum number of infective units per human dose, the origin of the substrates used in the preparation of the vaccine and whether the vaccine strains were derived by molecular methods;
— 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; and
— a statement that after the vaccine has been reconstituted, it should be used without delay, or if not used immediately, stored between 2 °C and 8 °C and in the dark for a maximum period defined by validation studies.

A.10 Distribution and shipping

The requirements of Good Manufacturing Practices for Biological Products (20) appropriate for a candidate vaccine should apply.

Shipments should be maintained at temperatures of 8 °C or below and packages should contain cold-chain monitors.

A.11 Storage and expiry date

The Requirements given in Good Manufacturing Practices for Biological Products (*22*) 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 national regulatory authority.

A.11.1 *Storage conditions*

Before being distributed by the manufacturing establishment or 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. After distribution, live tetravalent dengue vaccine should be stored at all times at a temperature not more than 8 °C. The maximum duration of storage should be fixed with the approval of the national regulatory authority and should be such as to ensure that the minimum titre specified on the label of the container (or package) will be maintained after release by the manufacturing establishment until the end of the shelf-life, if the conditions under which the vaccine is stored are in accordance with those stated on the label. The maximum duration of storage at 2–8 °C or below –20 °C may be specified.

A.11.2 *Expiry date*

An expiry date should be fixed and should relate to the date of the last satisfactory determination, performed in accordance with Part A, section 6.2, of virus concentration, i.e., the date on which the cell cultures were inoculated.

Part B. National control requirements

The national regulatory authority may give directions to manufacturers concerning the dengue virus strains to be used in candidate vaccine production and concerning the proposed human dose(s) to be tested.

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Document WHO/BS/04.1989 was prepared by Dr D. Wood, WHO, taking into account the comments from the December 2003 Consultation, for consideration by the fifty-fifth meeting of the Expert Committee on Biological Standardization, held in Geneva in 2004. Further changes were made to WHO/BS/04.1989 by the Expert Committee on Biological Standardization, resulting in the present document.

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WHO Technical Report Series, No. 932, 2006

Annex 2
Recommendations for the preparation, characterization and establishment of international and other biological reference standards (revised 2004)

The process whereby international biological reference standards are established, and the technical specifications with which they comply, are set out in this guidance document, which is intended to be scientific and advisory in nature.
The parts of each section printed in large type are definitive requirements for international biological reference standards. The parts of each section printed in smaller type are comments for additional guidance and are intended to provide further explanation of the text in large print.

Introduction

A core function of WHO, set out in its Constitution (Article 2), is to "develop, establish and promote international standards with respect to food, biological, pharmaceutical and similar products" as well as "to standardize diagnostic procedures as necessary". This responsibility is discharged in part by establishment of biological reference standards that form the basis of regulation and clinical dosing for biological medicines and also for regulation of in vitro diagnostic devices. The process whereby such international biological reference standards are established and the technical specifications with which they comply are set out in this guidance document, which is intended to be scientific and advisory in nature.

The provision of international biological reference standards makes a critically important contribution to high standards of efficacy, quality, purity and safety of many biological medicines used worldwide in the prevention, treatment or diagnosis of disease or conditions. Their use supports the application of the numerous biological and immunological assays used in the standardization and control of a wide range of biologicals including therapeutics, blood-derived products, vaccines and immunological products of traditional types as well as those derived from modern biotechnological approaches. They also have important applications in the standardization of materials and approaches used in medical diagnostics such as diagnosis of disease, monitoring therapy, blood safety and public health applications (e.g. monitoring immune status, screening for disease or susceptibility) or otherwise characterizing biological material from individuals.

WHO biological reference standards are widely used in the development, evaluation, standardization and control of products in industry, by regulatory authorities and also in biological research in academic and scientific organizations. They play a vital role in facilitating the transfer of laboratory science into clinical practice worldwide and the development of safe and effective biologicals.

There are special considerations and challenges which apply to the production and quality evaluation of biologicals, including the inherent variability of biological systems, variability of biological and immunological assays, and the potential for microbial contamination. The availability of WHO reference standards has made a major contribution to progress in the development and use of biologicals and in addressing these challenges.

In particular the reference standards have an essential role in the development of internationally agreed systems for measurement of

the biological and immunological activities of biological products. There is a wide variety of potential types of measurement: for example biological activity, immunological activity, quantity, biotypes and genetic types. In addition, for each measurement type, there are numerous possible variations on methodologies and reagents. Therefore, the purpose of the reference standards is to facilitate standardized characterization of biological samples, whatever the type of measurement or method used. Many WHO biological reference standards are designated as International Standards (IS) and provide the unique physical basis for the definition of International Units (IUs) of biological and/or immunological activity. Their use enables the achievement of consistency in the measurement of key attributes of biologicals, for example biological potency or immunological activity and, thus, the development of internationally agreed criteria for acceptability and standardization and control of products. It also provides the basis for the comparability of data from different sources in relation to specific products. Assays for markers of immunity (e.g. to infectious agents) are often defined in terms of agreed IUs of antibodies, providing a basis for an international consensus on the measurement of the immunological status of individuals or populations following vaccination or infection.

Some WHO biological reference standards do not carry the designation of ISs, but are nevertheless of great value in the standardization of assays applied to biological products and diagnostic materials.

The timely development of new reference materials and standards is a critically important aspect of harnessing new scientific developments for application in the form of safe and effective biologicals and securing improved world health.

This document provides an updated set of recommendations in relation to the development, evaluation, establishment and use of WHO biological reference materials.

The WHO Guidelines for the Preparation and Establishment of Reference standards for Biological Substances were first published in 1978 (*1*). The Guidelines were revised in 1986 (*2*) following decisions by the WHO Expert Committee on Biological Standardization to simplify the nomenclature of international biological reference standards (*3*) and that reference standards of human origin should be tested for evidence of possible contamination with human immunodeficiency viruses and hepatitis B virus (*4*). The Guidelines were revised again in 1990 (*5*) when a section was added on information to be provided in support of requests for adoption by the WHO

Expert Committee on Biological Standardization of international biological reference standards.

A number of developments have occurred since 1990. Partly because of scientific and technical advances, the range of materials classified as biological substances has altered: many older biologicals can be appropriately characterized by chemical and physical means and their WHO biological reference standards have been discontinued, while new groups of biological substances have been developed.

> Antibiotics came within the range of substances considered by WHO as biologicals at the time of their development, but now, for most antibiotic preparations, physicochemical testing, rather than biological testing, is accepted.
>
> On the other hand, new groups of substances have been developed as a result of advances in molecular biology. Biological reference standards are still needed when such materials are subjected to biological or immunological assay.

The need has been recognized for prompt availability of some reference standards that have not undergone the rigorous characterization and testing of international biological standards, leading to the establishment of a new group of WHO Reference Reagents which may act as interim standards (*6*, p.4). A priority-setting process for developing WHO biological reference standards has been published (*7*). The science of reference standard preparation and characterization has continued to evolve and the extent to which the principles for the characterization of reference standards in certain fields (*8*) can be applied to biological reference standards in general has been debated. Consequently, WHO has worked with the scientific community, national regulatory authorities, other standard-setting bodies and users through a series of consultations (*9–13*) to review the scientific basis of characterization of biological reference standards. As a result, the concepts used by WHO for biological standardization have been re-affirmed as appropriate to ensure the continued usefulness of this class of reference standards. During the consultation process it was however recognized that improved clarity in explaining the rationale for the principles used by WHO in biological standardization would be of benefit. This updated version of the Guidelines reflects these and other changes.

These Recommendations are divided into in three parts:

- General considerations address the scientific basis of biological standardization and the principles applied to WHO International Standards.
- Part A addresses the background to the need for an international biological reference standard, general considerations about

procurement and characterization of suitable material, factors to be taken into account in preparing a batch of a candidate reference standard and assessing its suitability, the testing and collaborative assay of the batch; and the information to be provided to WHO so that appropriate reference standards can be established by the WHO Expert Committee on Biological Standardization. A new section on quality assurance considerations has been included.
- Part B provides advice and guidance to regional and national regulatory authorities on the preparation and establishment of secondary biological reference standards. Such materials may be assigned values in IUs by assay against the corresponding WHO reference standard.

The parts of each section printed in smaller type are comments for additional guidance and are intended to provide further explanation of the main text.

General considerations

WHO biological reference standards comprise materials of complex composition that require biological or immunological assay for appropriate characterization. The biological or immunological assays used are usually comparative rather than absolute, and the reference standard is critical in defining the qualitative nature and the relative magnitude of the biological or immunological response. The published catalogue of WHO biological reference standards includes over 300 materials and is updated each time materials are added or removed from the list (*8*). Definitions used in the context of this document are given in section A.1.2.

The set of principles used by WHO for biological standardization are:

- that the reference standard should be assigned a value in arbitrary rather than absolute units, but there can be exceptions, where justified;
- that the unit is defined by a reference standard with a physical existence; and
- that in the establishment of the standard, a variety of methods is usually used and that the value assigned to the standard, and therefore the definition of the unit, is not necessarily dependent on a specific method of determination;

> Generally, WHO reference standards are established for analytes for which no reference measurement procedure ("reference method") has been agreed or established. In these cases the principles set out above will apply. Where a reference method has been defined and agreed, then

establishment of the standard and value assignment may be specifically based on that method.

- that the behaviour of the reference standard should resemble as closely as possible the behaviour of test samples in the assay systems used to test them;

  The general principle is that of "like versus like". Thus although it may not be necessary for the standard to be prepared in the same formulation or matrix as test samples, it is necessary that the dose–response characteristics of the standard are the same as those of tests samples. For example, the reference preparation for assaying the activity of factor VIII in a factor VIII concentrate is derived from factor VIII concentrate, rather than plasma.

  However, reference standards may be formulated in such a way as to preserve long-term stability of activity and, where the test systems are not adversely affected, formulations may differ from the formulation or matrices of substances to be examined. This principle means, for example, that the formulation of factor VIII standard does not match that of commercial products; that a monovalent vaccine reference preparation may be used to assay combination vaccine products, providing it is shown that the components of the combination vaccine do not interfere with the response of the monovalent reference preparation; and that the WHO reference standard for a diagnostic analyte is not necessarily formulated in plasma.

  However, there are an increasing number of exceptions to this generalization and some reference standards that do not resemble the biological substance are designed specifically for use in particular assays. This is particularly true for reference materials use in relation to modern molecular biological tests. An example is the International Standard for the mutant analysis by polymerase chain reaction and restriction-enzyme cleavage (MAPREC) assay of poliovirus type 3, that consists of synthetic DNA and is intended for assay of poliovirus mutants in vaccine preparations (*14*).

These principles derive from shared properties of complex macromolecular analytes:

— difficulties in unambiguously assigning a value in SI units, even to well-characterized proteins;
— the comparative rather than absolute nature of biological and immunological test procedures;
— the difficulty in quantitatively defining the analyte in terms of a biological response;
— the difficulty of defining reference methods; and
— the multifactorial nature of biological and immunological test methods, in which both quantitative and qualitative differences in activity may result from changes in the properties of the reference standard.

The implications of the factors listed above are twofold for the establishment of biological reference standards; firstly, that an analyte is in fact defined by the reference standard. This is distinct from the situation for some chemical reference standards, which can be fully characterized by physical or

chemical methods, where an analyte is defined by a reference method. Secondly, it cannot be proven analytically that, when a biological reference standard is replaced, the new material is identical to the old. The analyte is essentially redefined by the new reference standard. This means that the chain of traceability for the user is to the new reference standard. Where a reference standard is assigned an activity expressed in IU, every effort is made in the collaborative study design to ensure that the IU defined by a replacement reference standard is as similar as possible to the IU defined by the old reference standard so that continuity of the IU is maintained. For example, the IU of factor VIII activity in factor VIII concentrate was established in 1970, and the activity represented by this unit has been maintained through seven successive WHO international standards, providing a stable baseline over time with which to assess and compare the efficacy of factor VIII treatments for haemophilia.

The relative magnitude of biological responses forms the basis of the comparative procedures in which biological reference standards are used. It is desirable that biological reference preparations are not assigned values in terms of the absolute magnitude of the biological response, because this depends on a variety of conditions. For example, WHO collaborative studies typically show that an absolute biological response such as a 50% cell culture infectious dose ($CCID_{50}$) is more variable than the expression of the results as a relative potency in IU. In a few cases, for historical reasons, standards are defined in terms of a "consensus" absolute biological response and are not used for assignment of relative potencies. An example would be the International Standard for measles vaccine (live) which is assigned a value of 4.4 log 10 infectious units per ampoule. It should be noted that this leads to difficulties in maintaining continuity of the assigned unit when it becomes necessary to replace such a standard.

As a consequence of applying the above-mentioned principles, the activity or potency of a WHO biological reference standard is demonstrated by biological procedures and, where appropriate, is stated in arbitrary IUs. The reference preparation thus defines the numerical value of the unit and also has a role in qualitatively defining what is being measured (the analyte). It is implicit that the unit has no existence other than in relation to the reference preparation that defines it. Thus when stocks of a WHO biological reference preparation become depleted, high priority is given to the establishment of a replacement material (Appendix 1). Once a replacement standard has been established, the units defined by the previous standard formally cease to exist. In practice, every effort is made to assign a value to the new reference preparation that will preserve as closely as possible the value of the IU over time (continuity of the unit). This ensures that users do not experience differences from one year to the next (or one decade to the next) when using values derived from WHO biological reference preparations.

A further consequence of the principles given above is that several methods, and in particular those methods which are currently in use in the relevant field, are usually used in studies to characterize candidate biological reference standards. This approach embodies the recognition that it is usually not possible to select, on a rational basis, any single assay method from which to predict the biological activity of a preparation in humans.

It is recognized that biological materials may be shown to have different types of biological activity. Thus separate reference preparations

may be established as bioassay and immunoassay standards, or, different types of biological activity may be assigned to the same reference preparation.

> As an example of establishing separate reference preparations, the first International Standard for follicle-stimulating hormone, recombinant, for bioassay was established in 1995 with an activity of 138 IU/ampoule (*6*, pp. 26–27) and a different preparation was established in 1997 as the first International Standard for follicle-stimulating hormone, recombinant, for immunoassay with an activity of 60 IU/ampoule (*15*). Two reference standards are thus available for different uses and users need to ensure that the appropriate material is requested, depending on the intended use.
>
> As an example of assigning different types of biological activity to the same reference preparation, the second International Standard for low-molecular-weight heparin, established in 2003 (*16*), was assigned activities of 1097 IU of anti-Xa per ampoule and 326 IU of anti-IIa per ampoule.

It is also recognized that some international standards may be used for qualitative rather than quantitative purposes.

> This is particularly the case for some International Standard materials used in the in vitro diagnostics area. In such cases, an International Standard may be established without the assignment of an IU. In some cases no assignment of activity may be made or, alternatively, units of activity may be assigned in terms of a suitable property. For example, the first International Standard for MAPREC analysis of poliovirus type 3 is assigned a content of 0.9% 472-C nucleotide per ampoule (*14*).
>
> Previously, a reference standard established without an assigned IU was called an international biological reference reagent (5). However even at the time that these two categories were created, it was acknowledged that the distinction between the two was not always clear-cut (5). In this revision, the distinction is no longer maintained. It is essential at the outset of any study of a candidate biological reference standard to state clearly if the intended use of the material is for qualitative purposes, because this will significantly influence the study design.
>
> It may also be necessary to establish a panel of reference materials to aid evaluation of diagnostic tests. As an example, the Expert Committee on Biological Standardization established a reference panel of 10 individual genotypes of human immunodeficiency virus type 1 (HIV-1) to help assess the specificity of nucleic amplification technology based assays for HIV-1. The panel was established as the First International Reference Panel for HIV-1 genotypes and unitages were not assigned to the individual members of the panel (*17*).

The extent to which the general metrological topic of *measurement uncertainty,* as defined in the standard ISO 17511 (*8*) applies to biological reference standards has been raised in the light of new regulations from one region of the world concerning in vitro diagnostic devices. However, where international biological reference standards are to be assigned a value in arbitrary IUs, an uncertainty value is not given.

> As a consequence of defining the IU as a fraction of the contents of the container of the current International Standard and because the units

defined by any previous International Standard formally cease to exist, an uncertainty value is not given to the assigned IU.

Information on the variability observed during the course of a collaborative study to characterize the preparation is always documented in the collaborative study report, which is available to users. In a multimethod collaborative study, differences in potency estimates of the material using different methods may be apparent. Moreover, the nature of biological assays means that methods which are nominally the same in reality differ in many features. In the absence of a reference method, assumptions about an underlying true value (of potency of the material), or a probability distribution of values across methods, may not be valid. Summarizing all the components of variability observed in a collaborative study by quoting a single uncertainty value may not be helpful. The single uncertainty value does not reflect the variability between ampoules of the International Standard.

The memoranda accompanying reference standards should contain a statement of the coefficient of variation (CV) of fill of the preparation concerned to reflect ampoule-to-ampoule variation (*16*).

Another issue raised by ISO17511 (*8*) is the assumption of a metrological "hierarchy", in which SI units are of a higher metrological order than IU. A strict application would appear to imply that, where possible, procedures reporting SI units should be used to calibrate reference preparations regardless of any other considerations. The Expert Committee on Biological Standardization, after consideration of this issue (*11*), concluded that the choice of unit should be made on a case-by-case basis and reflect, and be based on, the biological and medical as well as the physicochemical information available.

Many biologicals exist in both active and inactive states, and the clinically relevant form of the analyte may depend on the diagnostic aim. For example, the active state of the placental hormone chorionic gonadotrophin (hCG) is the relevant molecule to measure in the diagnosis of pregnancy, whereas the biologically inactive free beta subunit (hCG-beta) is measured to diagnose choriocarcinoma. Generally, a measurement of biological activity is expressed in IU, whereas measurement of the amount of a protein or of a specific protein structure is expressed in SI. In this case there would be a compelling reason to relate the measurement of hCG to a unit of biological activity, and the measurement of hCG-beta to an SI unit of quantity. Accordingly WHO has established a reference preparation for hCG (currently the fourth International Standard, with an assigned content of 650 IU/ampoule) (7) and a reference preparation for hCG-beta (currently the first WHO Reference Reagent for immunoassay of hCG beta subunit, with an assigned content of 0.88 nmol/ampoule) (*18*). The former preparation was assigned a value based on bioassay, whereas the latter preparation had been extensively characterized by physicochemical and immunological methods and calibrated in nanomol by amino acid analysis.

Applying these considerations of the properties of biological analytes, and their measurement in the clinical situation allowed the WHO biological reference standard for hepatitis B surface antigen, assigned a value in arbitrary IU rather than in SI units, to be adopted by the medical devices sector of the European Commission as the standard required for the fulfilment of the so-called Common Technical Specifications (CTS) for in

vitro diagnostic devices. The Common Technical Specification document supporting the European (IVD) Medical Devices Directive 98/79 EC is a legally binding document within the 25 countries of the European Union.

Where it is appropriate for a WHO biological reference standard to be calibrated in SI units, the principles outlined in ISO 17511 (*8*) should be followed. This will necessitate the existence and use of an appropriate single reference method and an assignment of uncertainty, derived from calibration data. Such a reference method should not be a biological assay because the factors that affect the results of such assays are not fully understood. Where they are used, SI units assigned to biological reference standards should be derived from, and traceable to, physicochemical procedures.

The decision on the route of characterization to be followed for a WHO biological reference standard must be made and clearly stated at the outset of the study.

The concept of commutability also needs to be addressed. The way in which this is done may vary depending on the field of application. In the in vitro diagnostics area, the analyte may be a minor component of a complex biological matrix (e.g. blood). Matrix effects may have an important effect on the measurement.

In general terms, the concept of commutability seeks to establish the extent to which the reference standard is suitable to serve as a standard for the variety of different samples being assayed. This concept is considered in ISO15194 (*19*) to be an intrinsic property of the standard, and to require description. The way in which this is done may vary according to the intended application.

In the vaccine field, for example, International Standards for vaccines may be prepared without adjuvant, although vaccine preparations usually contain adjuvant. The applicability (or commutability) of the standard to such preparations will need to be established, either in the collaborative study, or by independent validation of assay methods.

Commutability in the in vitro diagnostics field is a consideration of how a reference preparation and samples to be examined compare in different assay methods, and is a property that is potentially affected by a wide range of factors including matrix (e.g. plasma or urine), binding proteins, plasma degradation and molecular variants of the analyte. A number of experimental approaches have been defined to determine this property, for example, a comparison of the ratio between the results of two procedures for the reference standard and for test samples. A commutable biological reference standard shows a similar behaviour to routine samples when different measurement procedures are applied. Generic specifications for similarity are difficult to formulate and are addressed on a case-by-case basis. Inclusion of real or surrogate clinical samples in the collaborative study may be a useful approach to enable evaluation of commutability. However, it should be noted that it can only be stated for the methods and samples studied, and a more extensive evaluation of commutability may require additional studies outside the WHO collaborative study.

In all fields of application, the extent to which commutability has been established should be clearly identified, as should any specific limitations of use identified in the commutability study.

In line with developments in other fields of reference standard characterization, a requirement to define what the biological reference standard measures is included in these revised Guidelines.

In other fields this is referred to as definition of the "measurand". For biological reference standards the measurand may be a protein structure, a biological activity or an immunological activity. In most cases, the definition of the measurand will be reflected in the procedures used to characterize and assign a value to the standard. Thus standards intended for calibration of bioassays will generally be characterized using bioassay procedures, those for immunoassays using immunoassay procedures, and so on.

Occasionally, and in particular in cases where the material is sufficiently well characterized to allow a complete physicochemical description, definition of the measurand may be achieved using a reference method distinct from the routine assay procedures. This approach is comparable to that used in clinical chemistry, for analytes which, although routinely assayed by immunoassays, may be measured as defined molecular entities by spectroscopic or other methods.

Examples include:

— the International Standard for somatropin (recombinant growth hormone), used as a primary calibrator for clinical immunoassays for growth hormone, is assigned a value in mg, traceable to amino-acid analysis of a physicochemically defined preparation; and
— synthetic DNA standards, used in the calibration of PCR assays, may be assigned a value based on phosphate determinations of a physicochemically defined synthetic polynucleotide.

Part A. Recommendations for the preparation, characterization and establishment of international biological reference standards

A.1 Introduction

A.1.1 *Background*

WHO establishes international biological reference standards for biological substances that are used in the prevention, treatment or diagnosis of human diseases or conditions. This is to enable their activity to be expressed in the same way throughout the world, in IU or other units, as appropriate and so provide a consistent basis for measurements.

The biological substances for which WHO establishes reference standards often consist of a heterogeneous mixture of isoforms, often not well characterized, and often in a complex matrix (such as serum/plasma).

A few biological reference standards have been established for preparations employed for the prevention, treatment or diagnosis of animal diseases of relevance to humans.

One example is anti-brucella arbortus serum.

International biological reference standards are not necessarily of high purity. However, when one preparation is replaced by another, every effort is made to ensure that the biological activity represented by one IU remains constant even if the specific activity of the preparation alters. International biological reference standards are usually available in relatively limited quantities and are intended to be used for the characterization and calibration of secondary preparations, whether of regional, national or more limited status; these secondary preparations are then used routinely.

A.1.2 *Definitions*

Reference standards are materials that are used as calibrators in assays. WHO provides reference standards for a range of substances which have been considered to be "biologicals" (see below), and which includes, but is not restricted to proteins, antigens, vaccines, antisera, blood products and nucleic acids. WHO reference standards are provided for the calibration of assays based on interactions of components of living systems, including those based on biological function, immunological reactivity, enzyme activation and enzyme amplification, and serve as global, "highest order" measurement standards for the analytes they define.

The definition of a medicinal substance, used in treatment, prevention or diagnosis, as a *"biological"* has been variously based on criteria related to its source, its amenability to characterization by physicochemical means alone, the requirement for biological assays, or on arbitrary systems of classification applied by regulatory authorities. For the purposes of WHO, including the present document, the list of substances considered to be *biologicals* is derived from their earlier definition as "substances which cannot be fully characterized by physicochemical means alone, and which therefore require the use of some form of bioassay". However, developments in the utility and applicability of physicochemical analytical methods, improved control of biological and biotechnology-based production methods, and an increased applicability of chemical synthesis to larger molecules, have made it effectively impossible to base a definition of a biological on any single criterion related to methods of analysis, source or method of production. Establishment of WHO measurement standards for any substance or class of substances is therefore based on an evaluation of current analytical methodologies, and where biological, immunological or enzymological methods are employed, an evaluation of the need for global measurement standards for calibration of these methods.

> For example, certain small proteins, such as cytokines and hormones, classed as "well-characterized", are now considered to be appropriately defined by physicochemical methods. Nonetheless, the need for biological measurement standards may be dictated by the need to define the specific activity of new products, or by the ongoing requirement to demonstrate specific activity of production batches. In the diagnostics field, the requirement for global measurement standards for otherwise well-characterized proteins and other macromolecules is driven by the routine use of comparative assay procedures such as immunoassays and nucleic acid amplification tests, and by the absence of reference methods for the definition of the analyte in absolute terms in reference materials.

The present document defines the major classes of WHO reference standard, and sets out guidelines and criteria for their preparation, characterization and establishment. The provisions of the document apply to each of the three classes of WHO reference standard described below, except where specific modifications are described.

The principal class of WHO reference standard is the *international biological measurement standard*. These are substances, classed as "biological" according to the criteria outlined above, which are provided to enable the results of *biological assay* or immunological assay procedures to be expressed in the same way throughout the world. The value assignment by WHO is in terms of an IU or another suitable unit. Provided that the candidate material has been shown to be suitable for its purpose, the unitage is attributed to a first international standard in an arbitrary manner after an international collaborative study has been completed. Activities in IUs are assigned to replacement international standards, where appropriate, by comparing them with the previous standard.

> An example of an International Standard that is assigned a unitage other than an IU is thromboplastin. The third International Standard for thromboplastin, human, recombinant, plain, is assigned an international sensitivity index of 0.940.

A *reference reagent* is a WHO reference standard, the activity of which is defined by WHO in terms of a unit. This category of reference standard is intended to be interim and replacement of the reference reagents is not envisaged. Sufficient information should have accrued in the period following establishment to allow consideration of the reference reagent as an International Standard. Only when a material established as an reference reagent is finally established as an International Standard will the potency be expressed in IU. It is expected that the formally assigned potency, in IU, following evaluation in an international collaborative study, be identical to the assigned unitage. Assignation of a different value would only be done on the basis of sound scientific reasons. Specific requirements for the establishment of reference reagents, as distinct from the general re-

quirements applicable to WHO International Biological Measurement Standards, are set out in Section A.8.

> The class of reference reagent was established in response to the speed of development of some new biological products (*6*). A need often arises from both regulatory and scientific considerations for reference standards with an official status conferred by WHO to be made available before the clinical utility of such new biological products becomes apparent. In such cases, the full programme of establishment of an International Standard may not be justified as the material may have limited use until the clinical utility of the biological product is established. In order to shorten the time between the preparation of a candidate material and its distribution, it is sufficient for a limited number of laboratories to examine a characterized product and agree to the assignment of potency as expressed in units. As a minimum, the bulk material used in the preparation should have been shown to retain biological activity consistent with the assigned unitage by a competent laboratory, for example the manufacturer, and this biological activity should have been confirmed by an independent laboratory, preferably a WHO collaborating laboratory. The candidate preparation should be shown to meet the specifications for filling and stability as defined in this document. The WHO collaborating centre should provide WHO with the necessary information on the source and characteristics of the preparation.
>
> Physicochemical characterization should be included if at all possible. It is not intended that such reference reagents should be product-specific.
>
> Such proposals may be submitted to WHO (see section A.7 for the format of collaborative study reports for reference standards).

An *international reference panel* is a group of reference materials established to collectively aid evaluation of assays or diagnostic tests. International reference panels comply in all respects with the general requirements for WHO reference standards set out in this document, except that in some cases it may not be necessary to assign unitages to each individual member of a panel.

A.1.3 ***Glossary***

In addition to the terms defined above, a number of the terms used throughout this document merit further explanation. The meaning of these terms in the context of this document is given in this glossary.

Baseline samples

Samples that are retained under optimal storage conditions to retain biological or immunological activity and that are used for comparison purposes. The baseline samples will need to be stored at a lower temperature than that used for the reference standard.

Biological tests (bioassay)

A procedure for the estimation of the nature or potency of a material by means of the reaction that follows its application to some elements of a living system (examples include animals, tissues, cells, receptors

and enzymes). The potency of the material being measured is often defined in IUs or, in some circumstances, may be defined in SI units, by comparison with the reaction of the system to that of a biological reference preparation.

Continuity
The concept that measurements in terms of the IU defined by a replacement reference standard are as similar as possible numerically to measurements in terms of the IU defined by the previous reference standard. This ensures that measurements made in biological and immunological tests can be compared over time.

Commutability
In general terms the concept of commutability seeks to establish the extent to which the reference standard is suitable to serve as a standard for the variety of samples being assayed. The way in which this is done may vary according to the intended application. Details of options for assessing commutability are given in the section on General considerations.

Immunological tests
A procedure that requires the use of antigens and/or antibodies to measure the analyte in a biological product or sample.

International unit
The unitage assigned by WHO to an International Biological Standard.

In vitro diagnostic devices
Tests for the detection of infectious agents, such as blood-borne pathogens that can be transmitted via blood and blood products, or conditions such as pregnancy.

Secondary reference standards
Reference standards established by regional or national authorities, or by other laboratories, that are calibrated against, and traceable to, the primary WHO materials and are intended for use in routine tests.

Traceability
Property of the result of a measurement or the value of a standard whereby it can be related to stated references, usually national or international standards, through an unbroken chain of comparisons.

Validation
Confirmation, through the provision of objective evidence, that requirements for a specific intended use or application have been fulfilled.

Uncertainty
An estimate attached to a test result that characterizes the range of values within which the true value is asserted to lie.

A.1.4 *Nomenclature issues for biological reference standards*

During the course of the WHO programme on biological standardization, some categories of reference standard have been established and later discontinued, for example, international reference preparations and international biological reference reagents.

> To ensure transparency and to avoid any confusion in use or in the literature, reference standards that were established in categories that are now obsolete retain their designation and have not been reclassified. However, when a preparation with an obsolete name is replaced, the new nomenclature should be used. The pathway from one class of name to the next should be clearly explained in the memorandum that accompanies the reference standard.

WHO standards for any given substance are identified by the assigned ordinate, as in the first International standard for . . . , the second International Standard, etc. It should be recognized that great care must be taken in the use of this system of nomenclature to avoid confusion with primary and secondary (e.g. working) standards. Where, as is usually the case, establishment of a second International Standard is accompanied by disestablishment of the first International Standard, it should be emphasized that the second standard has effectively replaced the first as the highest order reference standard, and critically, as the sole definition of the unit.

Although the source of some potential confusion about the hierarchy of standards, as outlined above, this system of nomenclature has proved useful in situations where the International Standard has been replaced on a regular basis, particularly in the unambiguous identification in the literature of which WHO standard published results are related to.

The year of establishment of a WHO standard should be given in the title of the document in which the preparation is decribed and also in catalogues listing WHO reference materials, on the label (section A.5.4) and in publications referring to the reference standard.

During the course of the WHO biological standardization programme, a number of examples have arisen of a native reference

standard (i.e. a reference standard derived from clinical material) being replaced by a recombinant material. In this case, the recombinant nature of the reference standard should be indicated in the title of the documents describing the preparation.

> An example would be the second International Standard for interferon B, fibroblast which was replaced by the third International Standard for interferon B, human, recombinant, glycoslyated (*16*).

When a native reference standard is replaced by a recombinant material a number of factors should be considered.

> It may be desirable to retain a sufficient stock of the replaced native material so that any future new or replacement recombinant standard may be calibrated against the native reference standard (*7*, p. 23). Consideration should also be given to whether the native material should be disestablished, or, in cases where native and recombinant material may be regarded as separate analytes, retained as a separate standard. Follicle stimulating hormone is an example of the application of the latter principle.

An international non-proprietary name (INN) may be in existence for the material for which a reference standard is established. Unless the reference standard is intended to be used to standardize only that material complying with the definition of the INN, the INN is not included in the title of the material, but is included in the memorandum sent out with that material.

> The point is illustrated by the example of tissue plasminogen activator (TPA). One preparation of recombinant TPA has been assigned the INN alteplase. Although the International Standard for TPA was prepared from alteplase, it is intended for use as a standard for TPA assays for TPA from all sources, and has therefore been named as the International Standard for TPA, rather than the International Standard for alteplase.

Where a reference standard is considered by the Expert Committee on Biological Standardization to be suitable for a restricted use only, this should be included in the name of the preparation.

> An example would be "the first International Standard for *a*, for immunoassay".

A.1.5 ***Purpose of these recommendations***

WHO designates certain centres as International Laboratories for Biological Standards. These laboratories have the responsibilities of serving as custodians and distributors of international biological reference standards. They have also been responsible for identifying needs for such reference standards, obtaining the materials and preparing and studying the batches either themselves or in collaboration with other laboratories. The expansion of the scope of work undertaken in biological standardization has led to a number of other laboratories and organizations becoming involved in making prepara-

tions that may ultimately be offered to WHO for consideration as biological standards. For this reason, the WHO Expert Committee on Biological Standardization has recommended that all proposals by international associations and other bodies for the establishment of international biological reference standards, should be submitted to WHO so as to avoid duplication of effort (*5*, p. 29).

Part A of these Recommendations is intended to reflect best established practice for the preparation, characterization and establishment of international biological reference standards. It therefore serves as guidance for any laboratory or organization that becomes involved in the preparation and testing of candidate materials intended for such a purpose.

Decisions on assigning priorities in developing WHO International Standards or interim reference standards should be based on the criteria specified in Appendix 1.

A.1.6 *Safety considerations*

Many biological materials, including those of human origin, intended for the preparation of an international biological reference standard must be considered as potentially hazardous. For reasons of safety in handling and use, the material itself or its original matrix (e.g. blood) must be obtained appropriately and screened for the presence of infection or other safety hazard. Blood should be obtained from donors who meet current international requirements (*20*). Non-human animal proteins should meet current WHO requirements (*21*).

> Screening will involve, as a minimum, the testing currently required for human blood and plasma, for example, for the presence of hepatitis B surface antigen and markers for HIV, hepatitis C virus and for other relevant pathogens (*21*).
>
> Tests for the presence of infectious markers (e.g. HIV markers) are not required for reference standards intended for the diagnosis of that infection (e.g. HIV infection), but suitable evidence of proper inactivation should be provided. The geographical area from which the source material is obtained should be recorded.

The actual or potential infectivity of biological materials of non-human origin, especially those derived from viruses or bacteria, should be taken into account. Suitable procedures may be applied to inactivate microorganisms or their components and the effectiveness of this inactivation should be demonstrated.

> The impact of any inactivation process on the fitness for purpose of the candidate standard should be investigated.

Furthermore, it is essential that appropriate precautions are taken to ensure that shipments of biological reference preparations comply

with international regulations on transport of infectious substances (*22*).

It is essential that suitable precautions are taken in the user laboratories during handling and disposal of biological materials to avoid possible infection. This is particularly important when the material is known or intended to be infectious. A safety data sheet (see Appendix 2) is provided with each reference standard.

A.2 Quality assurance

A.2.1 *Quality management system*

Biological reference standards should be obtained, processed, stored and dispatched under a defined quality management system. International recommendations are available from ISO (*23*, *24*). It is desirable that the quality management system be assessed as satisfactory by an independent body.

Other essential components of the process of standards development may be partly or entirely outside the control of the organizing or coordinating laboratory. These include:

— preparation and characterization of candidate materials in donor laboratories;
— characterization of candidate materials and trial formulations in testing laboratories;
— contribution of WHO and other consultative committees to study design;
— performance of testing by participants in collaborative studies; and
— review of data and formal establishment by the Expert Committee on Biological Standardization.

Although such activities may fall outside the possible scope of a formal quality system, it is strongly recommended that processes and documentation compliant with recognized quality standards, are, implemented and followed as far as possible.

> Managing organizations are encouraged to review continuously the entire process of standards development, from the initial sourcing of material through to the laboratories participating in the collaborative studies, with a view to bringing essential and controllable aspects of the process within defined quality management systems.

A.2.2 *Records*

It is essential that complete records are kept, in compliance with quality system requirements, relating to, inter alia:

— the background and proposals for preparation of the intended reference standard;
— the responsible persons and their defined roles;
— certificates of analysis of bulk materials intended for use as an international reference standard. If this is plasma or serum based, such information as is available about the donors should be included:

> Information about donors may include details of the donation centre, the gender and age of donors, and records of ethical approval for the donations.

— the procedures and tests which have been performed before, during and after filling into containers, including quality control tests for residual moisture and homogeneity;
— stability studies;
— raw data from collaborative studies;
— reports and recommendations;
— records of agreement or otherwise of participants; and
— storage, inventory and dispatch of the reference standard.

Such records form the basis of the IU as the fundamental unit of measurement for any given analyte. They should therefore be retained even after a standard is replaced, and should be kept until such time as the International Standard, and hence the IU, is *discontinued* and *not replaced*.

A.2.3 *Validation of methods*

The quality system should clearly identify critical equipment and technology and set out procedures for validating and maintaining its functionality. Such critical equipment includes, but is not restricted to:

— liquid handling equipment for dispensing into ampoules/vials;
— freeze-driers;
— isolators for sterile fills;
— ampoule/vial sealing equipment;
— equipment for carrying out in-process controls;
— air-filtration equipment for maintenance of sterile/clean rooms;
— sterilization, washing and water purification equipment; and
— storage equipment.

A.3 Assessment of need and procurement of materials

A.3.1 *Assessment of need*

International biological reference standards may be needed for:

— the assay or characterization of a biological product approved, or intended for approval, for use in medical practice and distributed in more than one country;

— the identification of a biological material of importance in medical or laboratory practice; or
— the calibration of regional, national or laboratory biological reference standards.

The WHO Expert Committee on Biological Standardization will not normally establish biological reference standards intended solely for research purposes.

> Exceptions are made where the availability of biological reference standards for research purposes may be of international public health significance. An example would be the WHO Reference Reagent for human brain, variant CJD, established to facilitate research to develop assays to detect the agent of variant Creutzfeldt–Jakob disease (vCJD) (*17*, pp.23–24).

When a need for an international biological reference standard is identified by another organization, it is essential that WHO should be informed of this and of whether that organization intends to proceed with preparation of the material, so as to avoid unnecessary duplication of effort. Coordination with other standard-setting bodies is important in this respect.

A decision tree (Appendix 1) aids allocation of priority to requests for new and replacement biological reference standards.

A.3.2 *Nature, source and storage of bulk material*

A fundamental tenet of biological standardization is that the behaviour of the reference standard should resemble as closely as possible that of the test samples in the assay systems used to test them. Choice of candidate materials should reflect this principle of assaying "like against like".

The bulk material selected should have a high degree of stability and a specific activity or concentration sufficient for the purposes of the assays or tests for which it is to be used. Although the material does not necessarily have to be of the highest purity, no other substances present should interfere with the procedures in which the material is to be employed.

> Generally speaking, the nature of the candidate material will reflect the current "state of the art" for any given analyte. Thus a therapeutic protein will generally be essentially pure, and will be provided with a certificate of release describing its specific biological activity, its physicochemical characterization, and its freedom from significant contaminants. Plasma products will be representative of current manufacturing capability, and in addition will be provided with certificates demonstrating compliance with current safety and ethical requirements. Vaccine preparations will represent current practice in preparation of microbial immunogens. Where the nature of the reference standard does not permit such detailed characterization

(e.g. plasma antibodies) then the characterization of the bulk material should, as a minimum, describe the biological activity in relation to the activity intended to be standardized.

The bulk material will usually be obtained from a single source. It may consist of part or all of a single batch. This may be difficult to achieve for standards derived from human plasma, in which case a small number of large samples, rather than a large number of small samples are the preferred source material.

For bulk materials manufactured by an industrial process, a certificate of analysis of the batch(es) should be provided by the donor of the bulk material. This information will not be disclosed to users without permission from the donor.

If it is necessary to prepare the bulk by pooling material from more than one batch or source, the procedure employed should ensure that the pooled material is mixed thoroughly and is homogeneous. For bulk liquids containing proteins, care should be taken to avoid denaturation during mixing. In addition to any studies that may have been made on the individual batches before pooling, the suitability of the homogeneous blend should be demonstrated.

When the bulk material used for, or the filled final batch of containers of, an international biological reference standard is of commercial origin, this fact should not be used for advertising purposes.

In order to serve as an international reference standard, a sufficient number of final filled containers of the bulk material should be available to meet the estimated demand, preferably for at least 10 years. The approaches to be taken for the eventual replacement of a standard should be considered when the proposals for preparation of the intended reference standard are drawn up.

To minimize any discontinuity of unitage, for example, in standards for complex diagnostic analytes where a wide range of assays of different specificities are supported, these approaches may include:

- obtaining and holding excess candidate bulk materials to allow replacement with identical material; or
- where long-term stability can be verified, extending the life of the standard by preparing larger fills, up to 20 000 ampoules.

The amount of bulk material needed for filling will depend on the estimated demand: a smaller quantity will suffice if the material is expected to be used by only a few laboratories.

The bulk material must be stored under suitable conditions before being distributed into final containers. Advice on optimum storage conditions should be obtained from the producer of the material before receipt of the batch. Sufficient samples to allow all necessary testing to be conducted should be removed from the bulk before it is

placed in storage. These samples should be stored under the same conditions as the bulk until they are tested.

Bulk materials may be stored in the dried form, provided that they can be dried without losing their biological activity and that, on being reconstituted, they retain adequate activity and have appropriate physical properties. Liquid bulk materials should usually be stored frozen and special precautions should be taken to achieve proper freezing. Liquid bulk materials may be stored at 2–8 °C provided that they are sterile or contain an antimicrobial preservative. In all cases, the containers of bulk material should be able to withstand the conditions of freezing, storage, thawing, opening and, if applicable, freeze-drying. In all cases, the storage conditions should ensure that the biological activity of the material is conserved.

A.4 Distribution into final containers

A.4.1 *General considerations*

An important requirement to be met by a batch of an international biological reference standard is that the material in every final container in the batch should be within specified limits, as defined below, in terms of composition, quantity, potency and stability.

In order that all the samples of a preparation are homogeneous, they should all be derived from the same homogeneous bulk, and should all be processed together in one working session. Processing should be performed in an environment with an appropriate low bioburden level. The bulk material is distributed, usually in liquid form to achieve high precision of fill, into a number of suitable containers. The contents of the containers are dried from the frozen state. This process may also be applied to insoluble solids that can be suspended in a suitable liquid. Materials that cannot be dried satisfactorily may, after dispensing, be stored as liquids provided that stability is retained under the storage conditions employed.

Suitable safety precautions should be taken to protect personnel and the environment from exposure to any potentially infectious or harmful material.

A.4.2 *Treatment of liquid bulk materials*

The choice of process and the extent of processing required for preparing the final bulk for filling will depend on whether the liquid bulk is a true solution, a colloid or a suspension. In all cases the processing should ensure that the product is homogeneous during filling, and measures should be taken at all stages to avoid contamination of the material. Liquids may have to be treated chemically or physically to

control microbial contamination or to remove particles or aggregates of active material. Water-soluble materials are dissolved at a suitable concentration in diluents, buffers or stabilizing solutions.

> These solutions should be prepared from water of a purity comparable to double glass distilled water, or higher, and pyrogen-free where appropriate.

If inclusion of an antimicrobial preservative is necessary, it should be one that will not affect the intended use of the preparation or volatilize during the drying process, and will not decrease the stability of the preparation.

> The choice of preservative is an important consideration because some countries place restrictions on acceptable preservatives. The choice of preservative should be justified and records of this retained.
>
> Cresol, phenol or sodium azide (which may form explosive compounds with metals) should not be used as preservatives in a preparation that is to be freeze-dried.

A biologically active substance is frequently present in a container of the reference standard in such small amounts that a bulking agent has to be present in the solution for filling to allow a visible freeze-dried plug of suitable size to be formed. In some instances, added materials are chosen to prevent or limit adsorption of the active substance on to the internal glass wall of the container and structural changes affecting biological activity that may occur during freeze-drying. No added substance should have adverse effects on the activity of the material or interfere with the assay or test for which the preparation is intended.

> If a protein carrier, such as human albumin is used, it should comply with current requirements for blood products for freedom from contamination (*20*, *21*) and proteolytic enzymes should be minimal. The use of certain sugars, particularly those with reducing groups (e.g. lactose) as bulking agents should be avoided as they can form stable complexes with amino groups in proteins.

Preliminary freeze-drying trials with extensive analysis of the dried material may be necessary to establish that an added substance has not affected the desired characteristics and potency of the active material. Such studies may include investigating the stability of the reconstituted trial preparation.

It is normal practice for the contents of each container to be sufficient for several analyses or assays. However, after reconstitution of a lyophilized material, it may be desirable to subdivide the resulting solution into several containers, each sufficient only for one or two assays. These containers must be stored in such a way that their contents remain unchanged. For scarce materials, the amount chosen to be filled into each container should take into account the need to conserve the material.

A.4.3 *Treatment of solid bulk materials*

It is recommended that filling solid bulk materials be avoided. However, materials that are insoluble in water or less stable in a freeze-dried form may have to be distributed into containers as powders. In such a case, special precautions should be taken to ensure that both the bulk material and the samples taken from it are homogeneous. Special mixing and sampling devices may be necessary.

A.4.4 *Quality of final containers*

Heat-sealed ampoules are used in preference to stoppered vials for international reference standards. A sealed glass ampoule does not allow exchange of gases and moisture with the atmosphere and the long-term stability of biological materials is generally much greater under these conditions.

Stoppered vials may be used for certain types of biological material, such as infectious preparations. Vials with rubber or elastomer stoppers may also be considered for the preparation of international reference standards that are used for qualitative purposes.

> Where possible, a small number of sealed glass ampoules of the material should also be prepared so that a baseline is available for checking stability should the need arise.

Containers should be of neutral (borosilicate) glass type I of appropriate quality, for example complying with the current requirements of the *European Pharmacopoeia* or the *US Pharmacopeia*. The glass must be free from stresses and the containers must be able to withstand sterilization by heat and temperature stresses, such as those resulting from rapid freezing to –80 °C. Actinic (brown) glass may be necessary for photosensitive materials but does not allow the contents to be seen clearly. If stoppered vials are used, the closures should be of appropriate quality, for example complying with current pharmacopoeial requirements for closures for injections.

> Containers and closures should not affect the stability of biological standards and this may be shown through validation studies.

The volume of the containers used depends on the amount of material required in each but a capacity of about 5 ml is generally suitable for fills up to 1 ml in volume.

A specification for the purchase of containers, and if necessary closures, should be established. Batches intended for use should be shown to conform to the specification. The shape and size of ampoules should be such that they can be filled easily, sealed by fusion of the glass without adverse effects on the contents, opened easily and their contents removed without difficulty.

It is advisable to use flat-bottomed ampoules for preparations to be lyophilized as this ensures good thermal conductivity between the bottom of the ampoule and the top of the shelf in the freeze-drier.

The containers should be cleaned by a process that does not involve use of a detergent. If the clean containers are to be stored at any time before filling, they should be placed in sealed dust-proof containers.

Cleaning without detergents may be done by heating in distilled water in an autoclave, by steaming in hydrochloric acid (20 g/l), or by acetic acid (2% v/v), or by ultrasonic treatment. The containers should then be rinsed several times with clean water and finally with distilled water. Steam admitted to autoclaves for cleaning or sterilization of glassware must be free from any volatile or non-volatile compounds that may be present as a result of the use of boiler-water additives. If steaming in hydrochloric acid is carried out in an autoclave, great care must be taken to remove residual traces of the acid from the autoclave afterwards. The washed containers should then be sterilized by dry heat in a clean, grease-free and silicone-free oven.

A.4.5 ***Distribution into containers***

A.4.5.1 *General considerations*

Containers are usually filled before labelling.

Each container in the batch either should be permanently marked with some form of in-process identification of the material being filled or a quality system should be in place to assure the separation of containers from different batches.

If containers are marked, the form of marking should not scratch the surface of the glass.

Containers should be filled from a single homogeneous bulk material. A liquid bulk should be stirred continuously during filling and held at constant temperature to ensure that homogeneity is maintained throughout the filling process. Exposure to direct sunlight should be avoided.

Filling should be carried out in a clean environment, for example a clean room or in a laminar-flow cabinet equipped with a high efficiency particulate arrestor (HEPA) filter to avoid any form of contamination.

Criteria for the quality of the air, or for the performance of air filtering systems should be written into the quality control specification, and relevant parameters monitored accordingly.

A sample for testing cannot be assayed more accurately than the reference standard against which it is compared. Because a reference standard in the dried state has to be reconstituted, thus introducing further variability, the precision of fill should be as high as possible and the coefficient of variation as low as possible to minimize

inaccuracy of assay. Assays of biological materials often differ considerably in their reproducibility. In setting a target precision of fill (maximum coefficient of variation) for a biological reference standard for quantitative measurement, regard should be paid to the reproducibility inherent in the assay procedure(s) in which it will be used.

> There is no formal pass or fail criterion for the production quality control parameters given below. The important criterion is fitness for purpose. Nevertheless the criteria specified below are expectations that are fulfilled by the vast majority of WHO biological reference standards.

A.4.5.2 *Liquid fills*

For each filling run, about 1–2% of the containers should be selected and weighed before and after filling to check the variation in the amount (volume or mass) filled into each container. The precision of fill or coefficient of variation (standard deviation divided by the mean) can be derived from the data obtained. The sample should be assessed for any consistent significant change in filling weights over the course of the process. The containers should be selected according to a procedure designed by a biometrician to ensure as far as possible that the sample is representative of the filling run.

The nature of a liquid influences the precision with which it can be dispensed for filling. A coefficient of variation not greater than 0.0025, that is 0.25%, is achievable for aqueous solutions with a 1-ml fill volume. However, more viscous liquids cannot usually be dispensed with this degree of precision. For liquids such as plasma or cellular materials, a coefficient of variation on a 1-ml fill of <1% is realistic. In cases where a reference standard is not to be freeze-dried, the volume filled into the container should be slightly in excess of the volume intended to be extracted by the user.

A.4.5.3 *Powder fills*

It is recommended that powder fills be avoided. Powder fills have been used in the past when the amount of material is not a limiting factor. They may be necessary for water-insoluble materials.

Most powders can be fed into containers by means of an automatic filler, but spoons of suitable size may also be used. Large variations in the amount per container may be unavoidable although this may be unimportant if an exact quantity of the contents is weighed out at the time of use. Special precautions will be necessary for solids that are hygroscopic or efflorescent as well as for those that may acquire an electrostatic charge and stick to the inside of the container.

A.5 Processing of filled containers

A.5.1 *General considerations*

International standards should be prepared using conditions in which it has been demonstrated that the biological activity and other significant properties of the material are not degraded or lost, that the activity of the final preparation is stable, and that the biological, physical and chemical properties of the standard are compatible with its intended use. Where the standard is a replacement, much of this information will already be available. However, new standards will require research and development to determine suitable conditions and formulations. This is achieved by carrying out and analysing small-scale trial fills, using conditions that mimic as closely as possible those used in the large-scale definitive fill. The programme of research and development should be clearly identified and recorded. The records should also specify details of baseline samples that have been retained for comparison purposes; samples should included both non-freeze-dried samples stored at –150°C (frozen baselines), and also freeze-dried samples stored at –150 °C.

The processing of filled containers should be completed under optimal conditions. It is essential to ensure that all the containers in a batch are processed together from the time of filling until the process is complete so that they are subjected to the same conditions at the same time. Only one material should be processed at a time in the freeze-drier because cross-contamination has been demonstrated to occur when more than one material is present.

Ampoules should only be sealed by fusion of the glass. If stoppered vials are used, it should be borne in mind that rubber or elastomer closures may be unsatisfactory for long-term storage because their physical properties may change and they may allow exchange of gases with the surroundings.

Samples should be taken at appropriate times during processing so that the baseline properties and potency of the material may be assessed. The samples, suitably sealed, should be preserved in the vapour phase of liquid nitrogen. They can be used to evaluate the effects of processing on the biological material and to confirm, for example, that there has been no change in composition or loss of biological activity.

A.5.2 *Processing of materials that are to be freeze-dried*

A.5.2.1 *Freezing*

The freezing process is very complex. When liquid containing water is frozen, pure ice forms first and the dissolved components become

progressively concentrated in the remaining solution. Electrolytes usually crystallize, but biological materials such as proteins and carbohydrates usually do not. Instead the viscosity of the solution increases to the point where it can be considered to be a glass and the whole liquid has become solid, i.e. completely frozen. The liquid in the containers should be frozen to a sufficiently low temperature to ensure that this condition is reached.

> This requires a temperature between about –20 °C for sodium chloride solution to about –50 °C for serum, but sometimes a liquid does not begin to freeze until well below its apparent "freezing temperature", a phenomenon known as "supercooling". The temperature at which any given solution is completely frozen should be determined in a preliminary study by a technique such as differential thermal analysis. Measurement of changes in electrical resistivity is less sensitive.

Depending on the rate of cooling and the temperature reached, the greatly increased salt concentration and pH changes in buffers may damage proteins and result in loss of their biological activity. Some antibodies, clotting factors and enzymes are known to denature during the freezing process. Thus, the rate and temperature at which the material is frozen are important in preserving its activity and solubility, and the most suitable conditions should be determined experimentally. Sometimes, the precise conditions for successful freeze-drying of a given liquid can only be deduced from experience with similar freeze-drying operations.

A.5.2.2 *Freeze-drying*

The filled containers are usually processed in a shelf freeze-drier. The containers are arranged, usually on trays from which the base can be withdrawn, on temperature-controlled shelves in an evacuated chamber. The temperature of the material in the containers should be recorded continuously. If heat is applied to the shelves during the process, care should be taken to ensure that it is applied uniformly. Water vapour sublimes from the ice in the frozen liquid and forms as ice on a condenser at a lower temperature than that of the shelves. Sublimation of water draws heat from the material in the containers which is replaced by heat from the shelves. Thermal conductivity is aided by removing the tray bases during the process.

The duration of the freeze-drying process should be validated and extend well beyond that found experimentally to be the minimum necessary because the temperature gradient between the walls of the chamber and the centre of a shelf can result in different rates of freeze-drying.

Between batches the freeze-drier should be cleaned and sterilized using validated procedures.

A.5.2.3 *Further drying*

The technical capabilities, such as low chamber pressure and low condenser temperature, of modern freeze-driers may reduce the need for further drying. Secondary desiccation was originally introduced because the earlier freeze-driers were less efficient and it was necessary to further reduce residual moisture. For some materials requiring very low residual moisture it may still be used.

A.5.2.4 *Sealing*

All lyophilized materials are hygroscopic. It is, therefore, essential that containers of the lyophilized reference standard are sealed, using validated methods, as soon as possible after drying is complete. Exposure to atmospheric moisture and oxygen should be kept to a minimum and should be the same for all containers in the batch. Devices are available to minimize uptake of moisture and oxygen (see for example, *25*, *26*).

The containers should be sealed in such a way as to preserve the integrity of the contents over the intended shelf-life of the preparation. Ampoules should be sealed by fusion of the glass by drawing either under vacuum or after filling with dry nitrogen.

> Ampoules can be tested individually for pinholes and cracks, usually by immersion in a bath of dye under reduced pressure. Ampoules containing a vacuum can be tested with a high frequency coil. All defective ampoules should be discarded. A suitable validation procedure may replace the need to test individual ampoules.
>
> Vials may be sealed with rubber or elastomer caps usually held in place with an aluminium cover. On occasion, screw-capped vials may be used.

The sealed containers should be labelled, stored at an appropriate temperature, and protected from light. The storage temperature is usually –20 °C but may be lower.

A.5.3 *Procedure where freeze-drying is not used*

When liquid or solid preparations are not to be freeze-dried, the containers holding them may be filled with an appropriate gas before sealing.

> This may be achieved by placing the filled containers in a chamber that is evacuated and filled with the pure, dry, inert gas. This process should be repeated several times to remove residual air and moisture. The containers are then sealed.

A.5.4 *Labelling*

Each container must be marked with an identifying code unique to the batch which permits positive identification throughout the filling process. Materials intended to serve as international biological

reference standards must not be labelled as such until they have been formally established by the WHO Expert Committee on Biological Standardization. Once this is done, each container in the batch should be labelled to show the following items of information:

- The name "World Health Organization".
- The name and status of the preparation in the form " International Standard (or Reference Reagent) for . . .".
- The year in which the reference standard was established by the WHO Expert Committee on Biological Standardization.
- The unique code allocated by the filling laboratory to enable the batch to be identified.
- The storage conditions recommended for the material.
- A statement that the material is not for use in humans.

If the size of the label permits, the following information may also be shown. If the size of the label is not sufficient, this information must be given in the instructions for use that accompany the standard:

- The potency or other parameter assigned to the reference standard. This is usually the number of IUs per container, but may be the mass of solid containing one IU; or the number of IUs per milligram.
- The name and address of the organization designated to hold and distribute the material.
- A statement that the material should be used as directed in the instructions for use (package insert, safety data sheet) accompanying the reference standard.

A.5.5 *Characterization of the final product in the container*

The residual moisture content and residual oxygen content of the final product in the container should be determined and evidence of freedom from microbial contamination obtained. The final product in the container should be tested and found satisfactory for potency or biological activity, as appropriate.

> There is no formal pass or fail criterion for the production quality control parameters given below. The essential criterion is fitness for purpose. Nevertheless the criteria specified below are expectations that are fulfilled by the vast majority of WHO biological reference standards.
>
> If a validated process is used, then tests are not needed on every standard.

A.5.5.1 *Residual moisture content*

This is determined using final containers to verify that drying has been adequate but not so excessive that the nature of the material has been changed.

The number of containers to be tested depends on the test methods to be used, and the lot size of the batch; the number is determined by reference to a predefined sampling plan. Various methods of determination are available of which the coulometric Karl Fischer method is the most widely used. Preparations with a moisture content of less than 1% (w/w) have shown adequate long-term stability. Higher values e.g. 5%, may be suitable in some cases. Because lyophilized materials are hygroscopic, precautions are necessary to avoid moisture uptake during the measurement procedure.

A.5.5.2 *Residual oxygen content*

The number of containers to be tested depends on the test methods to be used, and the lot size of the batch; the number is determined by reference to a predefined sampling plan. Residual oxygen is determined using at least three containers to confirm that the atmosphere within the container is inert and that the material is protected against oxidative change. Oxygen levels below 45 μmol/l when determined at atmospheric pressure using, for example, an oxygen fuel cell meter or mass spectrometer have been shown to ensure adequate long-term stability.

Residual oxygen determinations may not be needed on every new batch of ampoules if the process is adequately validated.

A.5.5.3 *Characteristics and potency or biological activity*

It is essential that the biological material in the container is demonstrated to have retained its integrity, composition and potency, or biological activity, using appropriate methods.

A.5.6 ***Stability of the final product***

Determination of the stability of reference standards, i.e. establishing the rate of loss of potency or activity, under a variety of conditions is desirable for three reasons:

- To provide an estimate of the length of time for which the reference standard will remain suitable for its intended purpose under its defined storage conditions.
- To define appropriate conditions for distribution of the reference standard to users.
- To determine the extent to which the reference standard will retain its activity over time after reconstitution.

In most cases, no independent scale of measurement is available for the reference standard which itself serves to define its unit of activity, and hence no direct method of estimating the rate of loss of potency of the reference standard under its defined storage conditions is possible. Indirect and approximate methods are therefore used for determining the rate of loss. These methods are generally based on the relationship between reaction rates and temperature given by the Arrhenius equation and a first-order reaction rate is frequently

assumed. Use of these methods requires that samples of the reference standard are stored at a range of elevated temperatures and tested for potency relative to samples of the reference standard stored at lower temperatures.

Kirkwood (*27–28*) has described an iterative procedure based on a maximum likelihood approach for estimation of the parameters of the equation relating degradation rate to temperature.

Many biological products appear to exhibit Arrhenius-type behaviour over a modest range of temperatures. However, as this relationship is approximate, particularly over wide temperature ranges, caution must be exercised in accepting the predicted rates of reaction. Reference standards are designed to be stable under defined storage conditions, and may also show no apparent loss of potency after storage at elevated temperatures. Experience has shown that reconstitution may be difficult for some reference standards after storage at high temperatures. Such factors must be taken into account when designing degradation studies. Lack of detectable degradation, and consequent lack of predicted stability, does not preclude the establishment of an International Standard.

An example of an International Standard where data appeared to follow the Arrhenius equation is the International Standard for thrombin, which gave a predicted loss of activity at –20 °C of less than 0.1% per year (*30*).

Data from the thermally accelerated degradation study may also be used to predict likely loss of activity at higher temperatures which may occur during distribution of the reference standard, and these data may be used to define appropriate conditions for distribution.

The selection of suitable analytical methods for monitoring the stability depends on the nature and intended use of the substance. The number of laboratories involved in stability studies is generally fewer than the number involved in the main collaborative study to assess the suitability of the candidate material.

Expiry dates are not assigned to biological reference standards, providing that long-term stability is predicted on the basis of existing data. In some circumstances further study, or monitoring on a case-by-case basis, taking into account data obtained from a thermally accelerated degradation study, may be recommended by the Expert Committee on Biological Standardization. If there is a change in storage conditions of the reference standard at the custodian laboratory, new stability studies are required. Some samples of the reference standard should be stored at temperatures lower than the customary storage temperature when the standard is initially prepared, to provide a low-temperature baseline for long-term stability studies.

For example, in an international collaborative study of the International Standard for thyroid stimulating hormone for immunoassay (*31*) samples were held at the storage temperature of –20 °C and baseline samples held at –150 °C for 7371 days (20 years); no difference was measured in the

stability of the samples held at the two temperatures. The loss of activity for the preparation, coded 81/565, stored continuously at –20 °C was 0.04% per year.

Available information about the stability of the material after reconstitution should be given to users. Other information on factors that may affect the properties of the reconstituted material, e.g. adsorption to particular types of container, should also be given.

This type of information will be limited because the conditions of reconstitution and storage generally cannot be extensively studied during collaborative studies.

Users are encouraged to send to WHO or the custodian laboratory, accounts of their experience in the use of the reference standard under routine laboratory conditions.

A.6 International collaborative studies

An international collaborative study must be carried out before any candidate biological reference standard can be considered for establishment by the WHO Expert Committee on Biological Standardization. The amount of work and resources required to carry out such a study should not be underestimated. For standardization projects carried out by WHO standardization laboratories or other WHO collaborating centres, WHO, through the Expert Committee on Biological Standardization, should be informed of the intention of the collaborating laboratory to undertake the work and have given agreement, in principle, to consider establishment of the candidate material, to avoid unnecessary or duplicated work. In agreeing, in principle, to the undertaking of work leading to the establishment of an International Standard, WHO may, either through Expert Committee on Biological Standardization or through the activities of working groups with vested responsibility for specific topics, make recommendation on the broad outline of studies to be pursued. The Expert Committee on Biological Standardization will not normally contribute to the specific detail of collaborative study design.

In some circumstances, WHO may establish collaborative links with other standardization organizations jointly to pursue specific standardization projects which have been prioritized and initiated independently. It is nonetheless desirable that through the Expert Committee on Biological Standardization, WHO prioritizes and endorses such projects before completion and establishment of the standard.

Collaborative studies should be organized by one or more scientist(s) familiar with the appropriate biological field, working closely with an

experienced biometrician, and according to the general principles set out below.

A.6.1 *Aims of a collaborative study*

The purpose of a collaborative study is to demonstrate that the candidate international biological reference standard is suitable for its intended use. A list of potential aims of the study are given below, but not all of these aims can be covered in a single study:

- Confirmation that the biological material has the properties and activity expected of it.
- Demonstration that the candidate reference standard is suitable for calibration of other reference standards or examination of preparations from a variety of manufacturers or sources.
- For reference standards intended for use in the diagnostics field, an assessment of commutability to clinical samples, where appropriate and feasible, should be considered.
- Comparison of two or more candidate materials.
- Assignment of a potency or other parameter to the contents of the containers.
- Whether different assay methods (e.g. bioassays and immunoassays, in vivo and in vitro assays) measure the same or different properties of a proposed reference standard. This may include assessment of the effects of biologically active contaminants.
- Comparison of a replacement batch with the current reference standard.
- Provision of a reference standard for a substance for which validated assay methods are not available.

  An example is the WHO human CJD reference panel that was established in 2003 and is intended for assay validation (*17*).

- Assessment of the stability of the proposed reference standard.
- Assessment of the molecular integrity and composition of the reference standard.

The aims of the study should be defined at the outset, if appropriate in consultation with WHO and potential participants.

An international collaborative study of a candidate biological reference standard is a scientific study designed to provide soundly based information for the Expert Committee on Biological Standardization on the characteristics of a proposed standard and its likely suitability for the intended use. Collaborative studies provide valuable scientific information about the materials studied and the assay systems in current use which could not be obtained by any one laboratory.

A.6.2 *Planning and design*

An international collaborative study for the characterization of a biological standard should be based on the principles of biological assay, designed according to sound statistical principles, and analysed and interpreted following sound statistical and biological principles. Although there is no generic design for a collaborative study, the principles set out below should be followed.

The details of the proposed collaborative study, and the underlying scientific rationale should, in all cases, be recorded, and these records retained throughout the time the standard is in use.

> Each study is unique and requires up-to-date scientific knowledge about the structure and function of the biological material, the nature of assays currently available, the availability of potential study materials and the availability of potential participants. This requires the participation of both a biological scientist and a biometrician, ideally with experience of such studies, to bring together experience of the biological material and the bioassays for it.
>
> The rationale for the proposed study design and the proposed statistical methods for analysis of the study should be outlined. It may be necessary to change both study design and methods of analysis to reflect the data which the participants are able to submit.

A key decision that will influence the study design is the choice of unit (IU or SI) intended to be assigned to the candidate reference standard. The choice of unit, and rationale for the choice, should be explicitly stated in the study protocol. If the study is of a replacement reference standard, the way in which continuity of the IU will be addressed is the key consideration in the study design and should be explicitly stated in the study protocol. The aim of continuity is that the IUs defined by a replacement reference standard are as similar as possible numerically to measurements in terms of the IUs defined by the previous reference standard. This is to ensure that measurements made in biological and immunological tests can be compared over time.

It is necessary to decide which samples will be examined in the study. For example, test materials other than the candidate reference standard(s) may have to be obtained. Inclusion of too many samples should be avoided.

> As an example, normal plasma pools may be included in studies of candidate reference standards for blood coagulation factors as a cross-check for the continuity of the IU. In such cases, the study report should provide details of the normal donor pools used to obtain the normal plasma pool.

The study should be designed so that each assay generates internal evidence allowing assessment of statistical validity (for example,

evidence of linearity and parallelism for parallel-line assays) and precision (*32*).

The number of participants will depend on the nature of the study, taking account of its aims, the number and type of assay systems included, the materials to be studied, the number of possible participants and their resources, and the capacity of the various assay systems.

> Where appropriate, working groups may be formed to facilitate the development of standards. Guidance may be provided on the methods to be used and the selection of laboratories.

If the study is complex in design, or new test procedures are being used, it may be necessary to include more participants than would be required, for example, for a study of a replacement standard using a well-defined pharmacopoeial assay method. If a new international biological reference standard is to be established with a defined unit of activity, a method for measuring the desired activity should exist already. If several assay methods are available, the material chosen should be suitable for use with as many of them as possible. The majority of studies are likely to include between five and 25 participants.

> An example of an international collaborative study conducted according to the principles outlined above, was the study to establish the International Standard for low molecular weight heparin (2003) (*16*).

Participants may be asked to carry out a specified minimum number of independent assays, or, if the assay procedure is known to be imprecise, a number sufficient to provide a mean estimate of acceptable precision. Duplicate assays may be requested. An independent assay is defined as one made using fresh dilutions from a newly opened container or a new weighing of each material and carried out on separate days. A duplicate assay is a repeat assay using the same solutions. Because it does not include all the variables of weighing and dilution, it is not truly independent.

A.6.3 *Participants and their role*

The participants may be national control laboratories, relevant manufacturers, academic or health care laboratories. The supplier of the material may also be a participant. Because the ultimate purpose of the study is the establishment of an international reference standard, competent laboratories representative of the six WHO regions should be included whenever possible.

Potential participants in the collaborative study should be given an outline of the aims of the study and a description of the materials to

be included. If it is intended that participants use the same assay method, a protocol for the procedure should be provided and sufficient time allowed for laboratories to become familiar with the method. Potential participants should be asked to indicate:

— the assay methods that they could use;
— whether they could compare the proposed number of materials in each assay;
— the number of assays that they could carry out;
— that they are willing to report their raw data using the reporting form supplied; and
— whether the laboratory operates under an accredited or other quality management system.

> The presence of a quality system does not guarantee the quality of the data submitted; the assessment of the collaborative study data is the key to data reliability.
>
> Prior to participation in a collaborative study, participants may be requested to undertake proficiency studies or tests with control samples.

Participants should also agree:

— to complete their studies within the period of time specified;
— to accept responsibility for safe handling and disposal of the materials provided;
— to use the materials provided for the purpose of the collaborative study only and not for independent research;

> Participants may be requested to sign a material transfer agreement, agreed between the donor of a sample for use in the collaborative study and WHO, as a condition of participation.

— not to publish information on a proposed international reference standard without the prior agreement of WHO, as premature publication before establishment of the material could cause scientific confusion.

> Participants should agree to a provisional plan for publication of the collaborative study, including proposed authorships, conditions, and provisions for anonymity, under which raw data from the study may be released for further analysis.

Participants will be asked to comment on the draft report of the collaborative study before its submission to WHO. The participants are listed, but the results from each participant are coded so as to retain anonymity.

A.6.4 *Materials to be included in the collaborative study*

Materials included in a collaborative study may include, in addition to the candidate standard(s), other standards in current use, coded

duplicate samples, typical samples for which the standard will be used (to assess commutability), samples that are known to be out of specification (e.g. samples that have failed a quality control test for a key parameter such as potency), or one or more dilutions of a sample included in the study.

Additional materials included in collaborative studies must be such that all the samples of a given preparation are within specified limits and stable during the time required for the study to be completed. To avoid introduction of bias, samples should be coded and labelled so that participants cannot identify materials and their sources or duplicate samples. Where appropriate, materials should be screened for freedom from infectious agents.

The materials should be distributed to the participants in accordance with current postal or air freight regulations (*22*). They should be securely packaged and appropriately labelled. If any materials are frozen, they should be packaged in insulated containers with sufficient coolant to last until they are delivered. They should be accompanied by directions for storage, handling and safe use and disposal. Participants should be requested to report the condition of the samples to the study organizer immediately upon receipt.

Temperature monitoring devices may be included with the shipment, or on the label of the standard.

If concerns about the condition of the samples are reported, the study organizer should decide as quickly as possible whether there is a need to ship replacement samples, and inform the participant of the decision concerning the condition of the samples.

A.6.5 *Reporting of results*

Each participant should be provided with a form on which to provide information on:

— the assay method(s) used, including details of the assay design and layout. This may also include details of the animals (species, strain, weight range, sex, pretreatment and method of randomization), or of other test materials (for example, organisms, cells, test kits or substrates);
— the nature of diluent solutions and the procedure for making dilutions of test and standard materials. This information is important for the calculation of results and the detection of causes of variation, bias or inaccuracy; and
— assay results given as raw (i.e. unprocessed) data. All data obtained should be reported, and an explanation must be given for proposed rejection of any data.

In addition, participants should provide their own statistical calculations for each assay as this helps to show whether they interpret their results in the same way as the biometrician who analyses all the results.

A.6.6 *Analysis of results*

Results from all participants are analysed by statistical methods described and considered appropriate by the biometrician responsible for the design of the study, who should be experienced in the statistical evaluation of the results of various types of assay. This analysis requires access to suitable computing facilities and statistical software. The results of each assay should be analysed separately and, as appropriate, the validity tested and the relative potency and precision calculated (for example, as means and 95% confidence intervals). Any questions about the results should be discussed promptly with the participant concerned.

The variability in results between assay methods, and between laboratories, should be described and assessed as part of the analysis. For example, an analysis of variance may be used to assess the statistical significance of differences between methods and laboratories. Other possible causes of variation, such as differences between candidate reference materials when more than one is included, should also be assessed. An assessment should be made of factors that may be the cause of significant heterogeneity of potency estimates, nonlinearity and differences in slopes. There is no general rule for the detection of outliers.

> Sources and causes of apparent outliers may not be consistent within assays, between assays, between laboratories or methods. Omission of any data should be taken into account in subsequent analysis.

As part of the overall study analysis, for each candidate preparation, the results of all the assays carried out by each participating laboratory, with each assay method, should be combined, where appropriate, and the potencies and confidence limits calculated. There is no generally applicable method for the combination of estimates.

> The methods to use for combination of estimates depend upon factors such as the intended use of the standard, information about assay systems, and the nature of the estimates and their distribution.
>
> The results of assays should also be displayed graphically, for example as histograms, as this may help to detect unusual features that could be overlooked in the study of numerical data alone.

A.6.7 *Report on a collaborative study*

A copy of the draft report is sent to each participant together with the code used to identify them. The participants should confirm that:

— their data have been correctly interpreted in the analysis;
— the proposed material is suitable to serve as a reference standard for the purpose defined; and
— the proposed unitage is appropriate.

The final report, after it has been amended where necessary and including a statement that the participants have agreed with it, is submitted to WHO. The information to be provided is outlined in section A.7 of these Recommendations.

> Any disagreement should be noted, together with any relevant critical comments, for further consideration by the Expert Committee on Biological Standardization.

The report of the collaborative study on a proposed international reference standard is the copyright property of WHO.

> The report published by WHO is assigned a document number and is intended for presentation to the Expert Committee on Biological Standardization as a working document. The study authors are strongly encouraged to submit a revised version of the report for publication in a peer-reviewed scientific journal. A manuscript submitted for publication should report the decision of the Expert Committee on Biological Standardization, and it is likely that the data and methods will need to be presented in a more concise manner than in the working document.

When the reference standard has been established, the report is used as the basis of the instruction leaflet for users that accompanies every dispatch of the material (a model is given in Appendix 2).

Data used to support the establishment of an international biological reference standard are made available to a user of the material either through reference to a scientific publication on the material or through the report provided to the Expert Committee on Biological Standardization to support the request to establish the material, or both.

> The working document (presented to the Expert Committee) describing the report of the study may be made available on the WHO web site (www.who.int/biologicals).

A.7 Detailed information to be provided to WHO

The following information should be provided in the report to WHO, in support of the submission of a request for adoption of a candidate preparation as an international biological measurement standard by the WHO Expert Committee on Biological Standardization. The information to be provided to support a proposal to establish an interim reference reagent is given in section A.7.6.

A.7.1 *Introduction*

The introduction should explain the background and need for an international reference standard. It should include:

— the name of the substance for which an international reference standard is proposed;
— a definition of the substance being measured (the "measurand");
— the rationale for the choice of units (IU or SI) being proposed;
— if the candidate reference standard is a replacement standard, the rationale for the approach taken to ensure continuity of the IU;
— the way in which the study has been designed to evaluate the fitness of purpose for the intended use of the reference standard, including, where appropriate, an assessment of commutability;
— whether the material is needed to standardize products for the prevention, treatment or diagnosis of disease;
— whether the material is subject to requirements for the manufacture and control of biological substances, is the subject of a monograph in a pharmacopoeia and is traded internationally;
— any recommendation by WHO or a recognized scientific organization that the material should be prepared;
— a review of methods currently used for the assay of similar materials, and the rationale for the choice of methods included in the study;
— the aims of the collaborative study and details of the participants;
— if a pilot study has been performed, appropriate details on the material used and the results; and
— if the reference standard is intended for use in the in vitro diagnostics field, the relationship of the approach used to the principles set out in ISO 17511 (*8*) where applicable.

A.7.2 *Bulk material and processing*

The following information should be provided on the bulk material and processing:

— description of the bulk material including its source, nature (including information about the donor(s) if relevant) and, where appropriate, its composition. This information may be supplemented by appropriate references from the literature, patent information or package inserts;
— details and results of safety and other chemical, physical and biological tests that have been performed;
— whether batches of bulk material were combined and, if so, the procedure used;
— the composition of the material filled, including buffers diluents, bulking agents or stabilizers;

— the identifying code of the candidate reference standard.
— the address of the facility where the bulk material was processed into final containers. If subcontractors have been used for any stage of the processing, the identity of the subcontractor(s) should be provided together with a list of the operations they carried out;
— full details of the processing operations (filling, lyophilization and sealing) and the dates on which they were performed;
— the number of containers used to estimate the precision of fill, the intervals at which weights were determined, and the results expressed as the coefficient of variation;
— evidence of validation of ampoule integrity after the sealing process;
— details of the gas under which the material was sealed, its purity, the method used to determine the residual oxygen content in the containers and the results obtained;
— the method used to determine the residual moisture content in the containers and the results obtained (as a percentage of the dry weight);
— details and results of other tests performed on the contents of the final containers;
— the number of final filled containers in the batch offered to WHO;
— the address of the intended place of storage and the name of the present custodian; and
— the storage conditions including temperature.

A.7.3 *Stability studies on the product in the final container*

The information on stability studies on the product in the final container should include:

— the name of the laboratory(ies) that obtained the stability data and details of the assay method(s) used to obtain them;
— the details of the stability study, including the number of assays carried out and the details of the samples assayed, including temperatures and duration of storage, and the results of assay of the activity remaining in each container after exposure to various temperatures, together with the 95% confidence intervals;

  The methods used for estimation of the 95% confidence intervals for the predicted percentage loss of activity per year (*33*) should be described.

— an assessment of the stability of the material;

  This may be based on the accelerated degradation studies, in the form of the predicted percentage loss of activity per year together with the 95% confidence intervals at the proposed storage temperature and any other appropriate temperature (e.g. +20 °C and/or +37 °C) which is similar to or higher than the conditions expected to be encountered during delivery of

the reference standards. In some cases other methods may be appropriate, such as real-time stability studies where the Arrhenius equation does not apply.

— an assessment of the stability of the reconstituted reference standard.

A.7.4 *The report of the collaborative study*

The following information should be provided on the collaborative study:

- The reason why a WHO biological standard is needed and the history of decisions of the Expert Committee on Biological Standardization or of WHO, if any, to support the need for the material.
- Planning and design of the collaborative study and descriptions of the nature of any other materials included in it.
- The assay methods used and which participants used them, described in such a way as to maintain blinding so that participants cannot be identified.
- For each assay method, the number of assays that each participant was asked to perform and the number actually carried out.
- A description of the statistical analysis carried out, including the way in which the linearity and parallelism of the dose–response curves were established and any problems that arose.
- Results obtained from the statistical analysis which should include:
  - — the numbers of valid and invalid results;
  - — the grounds for exclusion of any results (e.g. nonparallelism or nonlinearity);
  - — a comparison of assay results from materials tested by different assay methods, together with their interpretation and comments on particular factors, such as the frequency distribution of the estimates, differences in potency estimates and any observed factors which may account for these, and any differences observed between assay methods;
  - — for each laboratory using a given assay method, the within-assay variation and the overall between-assay variation should be stated where possible; and
  - — the overall estimates of relative potencies obtained by each assay method, calculated both with and without outlying results;

    The (raw) data should be available on request to WHO (Secretary, Expert Committee on Biological Standardization) for a period of at least 20 years, or longer if the standard is still in use.

- The final figure for the overall estimate of the potency of the proposed reference standard, comments on the validity of the over-

all estimate, and if appropriate, the 95% confidence intervals and the method of deriving them.
- In studies on proposed first international reference standards, an assessment of the degree to which the calculation of potencies relative to the proposed reference standard reduced differences between laboratories and between methods.

A.7.5 *Other information*

The report should also include:

- A recommendation for establishment of the material to serve as a reference standard together with any limitations on its use (e.g. suitability only for certain assay methods), together with a recommended potency in international or other relevant units.

  > The basis of assignment of units to the first International Standard for a material is the results of the collaborative study. Because the value assigned to the preparation is arbitrary in the case of IUs, it may be convenient to propose the value as a rounded number instead of a number derived by statistical analysis of the results. For replacement standards, the value proposed should ensure the continuity of the IU.

- A formal statement of the traceability path of the IU established by the proposed standard.
- A consideration of the relationship of the unit established by the proposed standard with previous units for the same material, including evaluation of the extent to which continuity of the IU has been maintained.
- A formal consideration of uncertainty, including a statement of the uncertainty of content derived from the variance of the fill, and an evaluation of the requirements of uncertainty statements in the context of the traceability path.
- An evaluation of the extent to which commutability has been demonstrated in the collaborative study.
- A list of the names and addresses of the participants. The coding used to refer to participants in the body of the report should not correspond to the order in which they are listed.
- Tables and histograms of the results of the collaborative study.
- A summary of the participants' comments on the report.
- Acknowledgements, summary and references.
- A copy of the proposed instruction leaflet and safety data sheet for users. It is recommended that a consistent format is used to ensure that no relevant information is omitted. A guide is given in Appendix 2.
- If requested, the detailed manufacturing records including results of in-process controls.

- If requested, detailed results of tests performed on the bulk and/or filled material.

A.7.6 *Report on a collaborative study on a proposed reference reagent*

The report on a collaborative study on a proposed reference reagent, which may be submitted for publication in a scientific journal, should include the following information:

— title;
— authors;
— summary (which includes the reason why the material is required; the number of laboratories and countries represented in the collaborative study; the aim of the study; the results; any comments; the stability of the proposed material; a proposal for adoption by the Expert Committee on Biological Standardization that states the code number of the preparation, and the proposed potency);
— introduction;
— the number of laboratories and countries represented in the collaborative study;
— materials (which, for the proposed reference reagent, should include the information specified in section A.7.2);
— stability of proposed interim reference reagent;
— assay methods;
— results (including the statistical analysis);
— discussion/conclusions;
— proposal (for adoption by the Expert Committee on Biological Standardization that states the code number of the preparation and the proposed potency);
— references (if any);
— participants (who, unless it has been agreed to the contrary, are referred to in the body of the report only by anonymous code numbers, which do not correspond to the order in which they are listed);
— tables and figures; and
— acknowledgements.

A.8 Establishment of an international biological reference standard

A preparation may be established as an international biological reference standard by the WHO when:

— the report on the collaborative study has been prepared, all participants have had the opportunity to comment on the report, and the report together with all comments have been presented to the Expert Committee on Biological Standardization;

— any queries raised by Working Groups or other groups requested by WHO to undertake a peer review of the proposals have been answered satisfactorily;
— all queries raised by members of the WHO Expert Advisory Panel on Biological Standardization after they have examined the information provided under Section A.7 have been answered satisfactorily; and
— the Expert Committee on Biological Standardization has come to an agreement based on the evidence provided and the expert recommendations for the material.

The decision of the Expert Committee is endorsed by the Director-General of WHO. A list of international biological reference standards is published from time to time in the WHO Technical Report Series and the current version is available on the WHO web site (www.who.int/biologicals). Reference standards that have been established or discontinued are included in an Annex to reports of meetings of the WHO Expert Committee on Biological Standardization. Catalogues are also available from custodian laboratories in printed and electronic form.

A.9 Storage and distribu tion of international biological reference standards

Custodian laboratories store and distribute international biological reference standards on behalf of WHO. The identity of the custodian laboratory for a particular reference standard is given in the above-mentioned list of reference standards. A key responsibility of the custodian laboratories is to maintain the integrity of the stored materials. The laboratories have comprehensive contingency plans to ensure that this integrity is maintained (*34*). Custodian laboratories are also encouraged to identify and maintain locations for off-site storage of sufficient numbers of each WHO International Standard to allow establishment of a replacement in the event of a catastrophe leading to the loss of or damage to the entire storage facility.

> These include monitoring of sample storage conditions and alarm systems with protocols and procedures in place to respond to alerts that are designed to maintain low-temperature storage of the preparations. Systems are also in place to avert accidental or intentional tampering with freezer or alarm settings. The laboratories have back-up emergency generators and provide relevant training for the personnel responsible for maintaining low-temperature storage of the reference standards.
>
> Custodianship of international biological reference standards requires considerable commitment and investment on behalf of the host institution.

Custodian laboratories ensure that appropriate precautions are taken to ensure that shipments of biological reference preparations comply, where appropriate, with international regulations on transport of infectious substances (*22*).

Part B. General considerations for the preparation, characterization and calibration of regional or national biological reference standards

B.1 Introduction

As supplies of an International Standard may be limited, regional and national authorities may consider preparing and establishing their own secondary reference standards, calibrated against and traceable to, the primary WHO materials, for wider use. Similarly, a manufacturer undertaking the assay of many batches of a biological product should usually establish a laboratory reference standard for routine use in these assays. The activities of such secondary preparations should be calibrated in IU by direct comparison with the international reference standard or, if necessary, by comparison with a regional or national reference standard. The amount of effort involved in setting up validated secondary reference standards should not be underestimated. For this reason, countries in a given region are advised to collaborate in the preparation of regional reference standards. The reference standards resulting from such collaboration are likely to have a wider application and duplication of effort is avoided or minimized.

International biological reference standards are distributed free of charge to national control laboratories and intergovernmental organizations for their intended purpose.

> International biological reference standards are usually not intended for use as working standards to be used every time a particular assay is performed.

If an international reference standard is not available from WHO, a regional or national authority may need to establish a reference standard and, if appropriate, define a unit of activity.

> An example is the *European Pharmacopoeia* unit of activity for some biological reference standards.

In preparing and establishing secondary reference standards, the principles and considerations set out in Part A apply, but some details may be modified. Particular points for consideration are set out below.

B.2 Assessment of need and procurement of material

The purposes for which a secondary reference standard may be needed are the same as for an international preparation, but the amount of the international reference standard available may be insufficient for frequent use, for example in routine testing of batches.

The purpose for which a material is required should be explained to the candidate supplier, usually a manufacturer. The composition of a secondary reference standard should resemble that of the materials to be assayed against it.

> Where possible, resemblance to the International Standard is desirable.

Frequently, materials will be supplied as final containers, often closed with rubber or elastomer stoppers. In this case, it is very important that the contents of the individual containers are homogeneous. Sometimes the regional or national laboratory will have to distribute a bulk material into final containers and will require the appropriate facilities to do so or should delegate this task to an appropriate body.

B.3 Distribution into and processing of final containers

Because regional, national and laboratory reference standards are likely to be used regularly and the batches may be replaced more frequently than those of international reference standards, it is acceptable to store them in stoppered vials rather than in sealed glass ampoules. The specifications for precision of fill, residual oxygen and moisture content should be sufficient to assure the suitability of the reference standard for its intended purpose. It is essential that the stability of the filled material is established and that it is sufficient for the time projected for the shelf-life of the batch. It is advisable to monitor stability through an appropriate programme and to re-check stability from time to time against the relevant international reference standard.

> Results of tests with the International Standard in the context of a stability-monitoring programme are of interest to WHO, and laboratories are encouraged to report the results to the Secretary, Expert Committee on Biological Standardization.

The requirements for labelling should be adapted to suit the context in which the material has been prepared and will be used.

B.4 Calibration

The calibration of a secondary reference material is a complex process and more extensive guidance than can be provided here is re-

quired. Considerations that need to be taken into account include, but are not necessarily restricted to:

- the higher order reference standards to which the regional or national standards are traceable, usually the WHO International Standard;
- compliance with regulatory requirements; calibration of secondary standards for therapeutic products should comply with local regulatory requirements whereas calibration of secondary standards for diagnostic use should follow the principles set out in ISO 17511 (*8*);
- whether an uncertainty value should be assigned; compliance with the requirements for metrological traceability will, in many cases, involve the use of restricted or single specified methods of analysis, and statements of uncertainty of the assigned unitage in terms of the International Standard, but there are exemptions as described by ISO 34;
- although the range of assay methods may be restricted, calibration will often involve a very large data set to minimize the uncertainty;
- how stability should be evaluated; stability testing is usually carried out using a programme for monitoring against the International Standard (rather than the predictive model used for establishment of the International Standard); and
- the need to verify the calibration obtained.

The number and geographical origin of the participants are likely to be more limited than for a global collaborative study to establish an International Standard. In some instances it may be sufficient to include as few as two participants, the body intending to establish the material and the supplier of the material. Great care should be taken to calibrate secondary reference standards as accurately as possible to avoid systematic bias in the estimation of potency. This may require a larger number of replicate assays.

Reports on collaborative studies to evaluate secondary reference standards should comply with the requirements of the organizing body. Final reports should be submitted to and retained by the organizing body. Instructions for use and safety information should be supplied to users with the reference standards.

Acknowledgements

The first draft of these revised recommendations was prepared by Dr D. Calam and reviewed at a consultation held from 26–27 June 2003 at the World Health Organization, Geneva. The consultation was attended by the following people:

Professor W.G. Van Aken, Amstelveen, the Netherlands; Dr T. Barrowcliffe, National Institute for Biological Standards and Control, Potters Bar, Herts., England; Dr A. Bristow, National Institute for Biological Standards and Control, Potters Bar, Herts., England; Dr D. Calam, Pewsey, Wiltshire, England; Dr W. Egan, Center for Biologics Evaluation and Research, Food and Drug Administraton, Rockville, MD, USA; Dr R. Gaines Das, National Institute for Biological Standards and Control, Potters Bar, Herts., England; Dr I. Hewlett, Center for Biologics Evaluation and Research, Food and Drug Administration; Rockville, MD, USA; Dr N. Lelie, Sanquin-CLB, Alkmaar, the Netherlands; Dr P. Minor, National Institute for Biological Standards and Control, Potters Bar, Herts., England; Dr M. Nübling, Paul Ehrlich Institute, Langen, Germany; Dr P. Phillips, National Institute for Biological Standards and Control, Potters Bar, Herts., England; Dr K. Zoon, Center for Cancer Research, National Cancer Institute/National Institutes of Health; Bethesda, MD, USA; Dr R. Büchel, Plasma Protein Therapeutics Association, Brussels, Belgium; Dr M. Duchêne, GlaxoSmithKline Biologicals, Rixensart, Belgium; Dr A. Eshkol, Serono International SA, Plan-les-Ouates, Switzerland; Dr D. Hendricks, Bayer Diagnostics, California, USA; Dr H. Parkes, Laboratory of Government Chemist, Middlesex, England; Professor I. Peake, University of Sheffield, Sheffield, England; Dr B.H. Phelps, Chiron Cooperation, Emeryville, USA; Professor I. Raw, Instituto Butantan, São Paulo, Brazil; Dr A. Sabouraud, Aventis Pasteur S.A., Marcy l'Etoile, France; Dr G.A. Scassellati, European Diagnostic Manufacturers, Brussels, Belgium; Mr J-M. Spieser, European Pharmacopoeia Commission, Strasbourg, France; Professor J. Thijssen, University Medical Center Utrecht, Utrecht, the Netherlands; Dr R. Wielgosz, International Bureau of Weight and Measures, Sèvres, France.

The draft WHO/BS/04.1995 was prepared by Dr D.J. Wood taking into account the comments from the above Consultation and a WHO Consultation on Global Measurement Standards and their use in the in vitro biological diagnostics field, held in Geneva from 7–8 June 2004, and comments from Dr A. Bristow and Dr R.E. Gaines Das, National Institute for Biological Standards and Control, Potters Bar, Herts., England and Dr A. Padilla, Dr G. Unger and Dr J. Shin, World Health Organization, Geneva, Switzerland.

The next draft of WHO/BS/04.1995 (27 October 2004) was prepared by Dr D.J. Wood, World Health Organization, Geneva, Switzerland and Dr A. Bristow and Mr A. Heath, National Institute for Biological Standards and Control, Potters Bar, Herts., England, taking into account the comments from a WHO Informal Consultation held in Geneva from 30 September–1 October 2004 attended by the following people:

Dr W.G. van Aken, Amstelveen, the Netherlands; Dr Y. Arakawa, National Institute of Infectious Diseases, Tokyo, Japan; Dr T. Barrowcliffe, National Institute for Biological Standards and Control, Potters Bar, Herts., England; Dr T. Bektimirov, Tarasevich State Institute for the Standardization and Control of Medical Biological Preparations, Moscow, Russian Federation; Dr A. Bristow, National Institute for Biological Standards and Control, Potters Bar, Herts., England; Dr E. Chaves Leal, INCQS/FIOCRUZ, Rio de Janeiro, Brazil; Dr T. Ciesiolka, Roche Diagnostics GmbH, Penzberg, Germany; Dr R. Decker, Hepatitis and AIDS Research, Deerfield, Illinois, USA; Dr W. Egan, Center for Biologics Evaluation and Research, Food and Drug Administration, Rockville, MD, USA; Dr S. Gairola, Serum Institute of India Ltd., Pune, India; Professor S.E. Grossberg, Medical College of Wisconsin, Milwaukee, WI, USA; Mr T. Hancox, International Organization for Standardization, Geneva, Switzerland; Mr A. Heath, National Institute for Biological Standards and Control, Potters Bar, Herts., England; Mrs T. Jivapaisarnpong, Ministry of Public Health, Nonthaburi, Thailand; Dr N. Lelie, Sanquin-Diagnostic Services, Alkmaar, the Netherlands; Dr J. Löwer, Paul-Ehrlich Institute, Langen, Germany; Ms R.M. Madej, Roche Molecular Systems, Pleasanton, CA, USA; Dr S. Marcovina, North

West Lipid Research Laboratories, University of Washington, USA; Dr P. Miede, Center for Biologics Evaluation and Research, Food and Drug Administration, Rockville, MD, USA; Dr H. Min, Korea Food and Drug Administraton, Seoul, Republic of Korea; Dr P. Phillips, National Institute for Biological Standards and Control, Potters Bar, Herts., England; Dr G. Schild, Imperial College School of Medicine, London, England; Dr J. Sokhey, Regional Office for South-East Asia, New Delhi, India; Mr J-M. Spieser, European Pharmacopoeia Commission, Strasbourg, France; Dr R. Wielgosz, International Bureau of Weights and Measures, Sèvres, France; Dr Tiequn Zhou, National Institute for the Control of Pharmaceutical and Biological Products, Beijing, People's Republic of China; Dr K. Zoon, National Institute of Health, Rockville, MD, USA.

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Appendix 1

Considerations for assignment of priorities to development of WHO International Biological Measurement Standards or Reference Reagents

Based on WHO Technical Report Series, No. 904, Annex 3.

Type of standard	Decision point	Guidance	Comments
International Standard	Is the proposed material a replacement or a new standard?	A replacement standard generally has a higher priority than a new standard.	A higher priority for a new standard can be justified where the material is expected to have a high impact, based on the considerations below.
	Is the proposed material to be used to standardize an approved medicine, or an established in vitro diagnostic method, rather than an investigational medicine or investigational method?	A candidate standard for an approved medicine or established method generally has the higher priority.	A higher priority for a standard for an investigational product or method can be justified where the product or method is in late-stage development.
	Is the proposed material a potential standard for more than one product or method?	A candidate standard for more than one product or method will generally have a higher priority than a product-specific or method-specific standard.	
	Is the proposed material to be used to standardize a product or in vitro diagnostic method of public health importance?	A candidate standard for a product or method of major public health importance will generally have higher priority than standards for other medical indications.	

Type of standard	Decision point	Guidance	Comments
	Is the proposed material to be used to standardize a product or method of global importance?	A candidate standard for a product or method of global importance will generally have higher priority than standards of regional importance.	A higher priority for a regional standard can be justified if the material is expected to have a high public health impact.
Reference reagent	Is the proposed material to be used to standardize a product or method for which the clinical utility is not yet apparent, or methods are not yet agreed?	A candidate reference reagent for which an international need exists from both regulatory and scientific considerations will have a higher priority than a reagent for which no such need exists.	

Appendix 2

Information to be included in instruction leaflets and safety data sheets for users of international or other biological reference standards

It is strongly advised that these leaflets and data sheets are prepared in a standard format.

> Publication of instruction leaflets on the WHO web site and also on the web site of the custodian laboratory is encouraged.

The package insert or instructions for use accompanying an international biological reference standard should include the following information:

- The name and address of the custodian laboratory and of the distributor if different.
- The name of the reference standard and its identifying code.
- The status of the material (International Standard or interim Reference Reagent) and the year of establishment.
- The defined potency or other parameter, together with a reference to the relevant WHO Expert Committee and collaborative study reports.
- Citation of the report submitted to the Expert Committee on Biological Standardization that supported the establishment of the standard and citation of any publications in the scientific literature describing the characterization of the reference standard:

  > The report submitted to the Expert Committee on Biological Standardization that supported the establishment of the standard may also be distributed together with the instruction leaflet and safety data sheet.

- Details of preparation of the material relevant to its use that, where appropriate, may be conveyed to the user with the agreement of the provider of the source material, such as
  - — details of the nature and formulation of the filled material;
  - — mean fill volume or mass with number of containers tested and coefficient of variation; and
  - — residual moisture and oxygen with number of containers tested.
- Recommended storage temperature and time. Because the distributor has no control over the conditions under which the reference standard is held after receipt, an instruction to use the material as soon as possible after receipt is advisable.
- Where appropriate, the method of reconstitution with the period of use and storage conditions after reconstitution.

- The intended use of the material:

  For standards intended for use with in vitro diagnostic devices, detailed information may be provided, where available, to assist users to document traceability to the reference standard. This may be in the form of a protocol that evaluates the lack of matrix effect in newly developed methods; evaluates the linearity of the reference standards in the system under evaluation; specifies the procedure for transfer of the assigned value of the reference standard to the user's calibrators; and provides information to validate the accuracy and the precision of the system under evaluation.

- Directions for safe use and disposal of the reference standard before and after reconstitution.
- A statement that the material is not for administration to humans.
- Any disclaimers over liability concerning use of the material.

Most of this information is required in the instructions for use of secondary reference standards.

Safety data sheet

The following information should be given in a safety data sheet:

- The name and address of the custodian laboratory and the distributor if different.
- The name of the reference standard and its identifying code.
- The status of the material (International Standard or Reference Reagent) and year of establishment.
- The physical nature of the material and, if freeze-dried, a statement that it is hygroscopic.
- Any hazards on exposure to the contents of the container.
- For material that is potentially infectious, a statement to this effect together with details and results of the testing for infectious agents that has been performed.
- For pathogenic material, a statement to this effect.
- Instructions for safe handling and disposal, including action to be taken with spillages.
- Instructions on action to be taken if someone is exposed to the material by direct contact including skin contact, ingestion and accidental injection.

The same information is required for a secondary reference standard.

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Annex 3
Recommendations, guidelines and other documents for biological substances used in medicine

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

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

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

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

Marketing and Dissemination
World Health Organization
1211 Geneva 27
Switzerland
Telephone: +41 22 79 12 476
Fax: +41 22 79 14 857
email: publications@who.int

Individual recommendations and guidelines may be obtained free of charge as offprints by writing to:

Quality Assurance and Safety of Biologicals
Department of Immunization, Vaccines and Biologicals
World Health Organization
1211 Geneva 27
Switzerland

[a] Abbreviated in the following pages as TRS.

Recommendations, Guidelines and other documents

Recommendations, Guidelines and other documents	Reference
Acellular pertussis component of monovalent or combined vaccines	Adopted 1996, TRS 878 (1998)
Animal cells, use of, as in vitro substrates for the production of biologicals	Revised 1996, TRS 878 (1998); Addendum 2003, TRS 927 (2005)
Antitumour antibiotics, guidelines for quality assessment	TRS 658 (1981)
Biological standardization and control: a scientific review commissioned by the UK National Biological Standards Board (1997)	Unpublished document WHO/BLG/97.1
Biological substances: international standards and reference reagents, guidelines for the preparation, characterization and establishment	Revised 2004, TRS 932 (2005)
BCG vaccine, dried	Revised 1985, TRS 745 (1987); Amendment 1987, TRS 771 (1988)
Biological products prepared by recombinant DNA technology	Adopted 1990, TRS 814 (1991)
Blood, blood components and plasma derivatives: collection, processing and quality control	Revised 1992, TRS 840 (1994)
Blood plasma products, human: viral inactivation and removal procedures	Adopted 2001, TRS 924 (2004)
Cholera vaccine (inactivated, oral)	Adopted 2001, TRS 924 (2004)
Dengue virus vaccine (tetravalent, live)	Adopted 2004, TRS 932 (2005)
Diphtheria, tetanus, pertussis and combined vaccines	Revised 1989, TRS 800 (1990); Addendum 2003, TRS 927 (2005)
DNA vaccines	Adopted 1996, TRS 878 (1998)
Good manufacturing practices for biological products	Adopted 1991, TRS 822 (1992)
Haemophilus influenzae type b conjugate vaccines	Revised 1998, TRS 897 (2000)
Haemorrhagic fever with renal syndrome (HFRS) vaccine (inactivated)	Adopted 1993, TRS 848 (1994)
Hepatitis A vaccine (inactivated)	Adopted 1994, TRS 858 (1995)
Hepatitis B vaccine prepared from plasma	Revised 1987, TRS 771 (1988)
Hepatitis B vaccines made by recombinant DNA techniques	Adopted 1988, TRS 786 (1989); Amendment 1997, TRS 889 (1999)
Hormones and other substances in community health care, development of national assay services	TRS 565 (1975)

Recommendations, Guidelines and other documents	Reference
Human hormones and their binding proteins, recommendations for the assessment of binding-assay systems (including immunoassay and receptor assay systems). (A guide to the formulation of requirements for reagents and assay kits for the above assays and notes on cytochemical bioassay systems.)	TRS 565 (1975)
Human interferons made by recombinant DNA techniques	Adopted 1987, TRS 771 (1988)
Human interferons prepared from lymphoblastoid cells	Adopted 1988, TRS 786 (1989)
Influenza vaccine (inactivated)	Revised 2003, TRS 927 (2005)
Influenza vaccine (live)	Adopted 1978, TRS 638 (1979)
Japanese encephalitis vaccine (inactivated) for human use	Adopted 1987, TRS 771 (1988)
Japanese encephalitis vaccine (live) for human use	Adopted 2000, TRS 910 (2002)
Louse-borne human typhus vaccine (live)	Adopted 1982, TRS 687 (1983)
Measles, mumps and rubella vaccines and combined vaccine (live)	Adopted 1992, TRS 848 (1994); Note TRS 848 (1994)
Meningococcal polysaccharide vaccine	Adopted 1975, TRS 594 (1976); Addendum 1980, TRS 658 (1981); Amendment 1999, TRS 904 (2002)
Meningococcal C conjugate vaccines	Adopted 2001, TRS 924 (2004); Addendum 2003, TRS 926 (2004)
Monoclonal antibodies	Adopted 1991, TRS 822 (1992)
Pneumococcal conjugate vaccines	Adopted 2003, TRS 927 (2005)
Poliomyelitis vaccine (inactivated)	Revised 2000, TRS 910 (2002); Amendment 2003, TRS 926 (2004)
Poliomyelitis vaccine (inactivated): guidelines for the safe production and quality control of inactivated poliovirus manufactured from wild polioviruses	Adopted 2003, TRS 926 (2004)
Poliomyelitis vaccine, oral	Revised 1999, TRS 904 (2002); Addendum 2000, TRS 910 (2002)
Quality assurance for biological products, guidelines for national authorities	Adopted 1991, TRS 822 (1992)
Rabies vaccine (inactivated) for human use, produced in continuous cell lines	Adopted 1986, TRS 760 (1987); Amendment 1992, TRS 840 (1994)

Recommendations, Guidelines and other documents	Reference
Rabies vaccine for human use	Revised 1980, TRS 658 (1981); Amendment 1992, TRS 840 (1994)
Regulation and licensing of biological products in countries with newly developing regulatory authorities	Adopted 1994, TRS 858 (1995)
Rift valley fever vaccine	Adopted 1981, TRS 673 (1982)
Smallpox vaccine	Revised 2003, TRS 926 (2004)
Sterility of biological substances	Revised 1973, TRS 530 (1973); Amendment 1995, TRS 872 (1998)
Synthetic peptide vaccines	Adopted 1997, TRS 889 (1999)
Thiomersal for vaccines: regulatory expectations for elimination, reduction or removal	Adopted 2003, TRS 926 (2004)
Thromboplastins and plasma used to control oral anticoagulant therapy	Revised 1997, TRS 889 (1999)
Tick-borne encephalitis vaccine (inactivated)	Adopted 1997, TRS 889 (1999)
Transmissible spongiform encephalopathies in relation to biological and pharmaceutical products, guidelines	Unpublished document WHO/BCT/QSD/2003.01
Tuberculins	Revised 1985, TRS 745 (1987)
Typhoid vaccine	Adopted 1966, TRS 361 (1967)
Vaccines, clinical evaluation: regulatory expectations	Adopted 2001, TRS 924 (2004)
Vaccines, nonclinical evaluation	Adopted 2003, TRS 926 (2004)
Varicella vaccine (live)	Revised 1993, TRS 848 (1994)
Vi polysaccharide typhoid vaccine	Adopted 1992, TRS 840 (1994)
Virus vaccines, summary protocol for the batch release	Adopted 1991, TRS 822 (1992)
Yellow fever vaccine	Revised 1995, TRS 872 (1998)
Yellow fever vaccine, laboratories approved by WHO for the production of	Revised 1995, TRS 872 (1998)
Yellow fever virus, production and testing of WHO primary seed lot 213-77 and reference batch 168-73	TRS 745 (1987)

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Annex 4
Biological substances: International Standards and Reference Reagents

A list of International Standards and Reference Reagents for biological substances was issued in WHO Technical Report Series, No. 897, 2000 (Annex 4) and an updated version is available on the Internet at http://www.who.int/biologicals. Copies of the list may be obtained from appointed sales agents for WHO publications or from: Distribution and Sales, World Health Organization, 1211 Geneva 27, Switzerland.

These substances are held and distributed by the International Laboratory for Biological Standards, National Institute for Biological Standards and Control, Potters Bar, Herts., EN6 3QG, England.

At its meeting in November 2004, the Expert Committee made the following changes to the previous list.

Preparations Additions	Activity	Status
Antigens and related substances		
Poliomyelitis vaccine, oral	7.51 $\log_{10}$ $TCID_{50}$/ml poliovirus type 1 6.51 $\log_{10}$ $TCID_{50}$/ml poliovirus type 2 6.87 $\log_{10}$ $TCID_{50}$/ml poliovirus type 3 7.66 $\log_{10}$ $TCID_{50}$/ml total poliovirus content	Second International Standard, 2004
Pertussis serotype 2, typing serum	No assigned value	First Reference Reagent, 2004
Pertussis serotype 3, typing serum	No assigned value	First Reference Reagent, 2004
Blood products and related substances		
Anti-D blood typing serum, for minimum potency of blood grouping reagents	No assigned value	First International Standard, 2004
Factor V Leiden, human	No assigned value	First International Genetic Reference Panel, 2004

Preparations Additions	Activity	Status
Factor XIII, plasma, human	0.91 IU/ampoule	First International Standard, 2004
Immunoglobulin, intravenous: anti-D positive control	No assigned value	First Reference Reagent, 2004
Immunoglobulin, intravenous, anti-D negative control	No assigned value	First Reference Reagent, 2004
Disestablishment ***Antigens and related substances***		
Hepatitis b vaccine, plasma derived	No assigned value	First International Reference Reagent