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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.

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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.

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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.