World Health Organization

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Recommendations for the preparation, characterization, establishment and use of WHO biological reference materials

Proposed revision of Annex 2 of WHO Technical Report Series, No. 932

NOTE:

This draft document has been prepared for the purpose of inviting comments and suggestions on the proposals contained therein which will then be considered by the WHO Expert Committee on Biological Standardization (ECBS). The distribution of this draft document is intended to provide information on the proposed revision of Annex 2 of WHO Technical Report Series, No. 932 to a broad audience and to ensure the transparency of the revision process.

Written comments proposing modifications to this text MUST be received by 6 February 2026 using the Comment Form available separately and should be addressed to the Department of Health Products Policy and Standards, World Health Organization, 20 Avenue Appia, 1211 Geneva 27, Switzerland. Comments may also be submitted electronically to the Responsible Officer: **Dr Tiequn Zhou** at: zhout@who.int.

The outcome of the deliberations of the ECBS will be published in the WHO Technical Report Series. The final agreed formulation of the document will be edited to be in conformity with the second edition of the *WHO style guide* (KMS/WHP/13.1).

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Abbreviations

BS	Biological Standardization (summary report)
CRM	certified reference material
DNA	deoxyribonucleic acid
ECBS	WHO Expert Committee on Biological Standardization
HBV	hepatitis B virus
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HPLC	high-performance liquid chromatography
IFU	instructions for use
INN	international nonproprietary name(s)
IPC	internal process control(s)
IRP	WHO International Reference Panel(s)
IRR	WHO International Reference Reagent(s)
IS	WHO International Standard(s)
ISO	International Organization for Standardization
IU	International Unit(s)
IVD	in vitro diagnostic
NAT	nucleic acid amplification technique
PCR	polymerase chain reaction
RMP	reference measurement procedure
RNA	ribonucleic acid
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
SI	International System of Units
U	Unit(s)
WHO BRM	WHO biological reference material(s)
WHO CC	WHO collaborating centre

Introduction

A core function of the World Health Organization (WHO), as set out in Article 2 of its Constitution, is to "develop, establish and promote international standards with respect to food, biological, pharmaceutical and similar products" and to "standardize diagnostic procedures as necessary" *(1)*. This responsibility is discharged in part through the establishment of WHO biological reference materials (WHO BRM) that form the basis of regulation and clinical dosing for biological products, and the regulation and evaluation of in vitro diagnostics (IVDs). The timely development and establishment of WHO BRM are therefore critically important in ensuring the development and availability of safe and effective biological products, and thus in improving global health. The process whereby such reference materials are prepared, characterized and established, and the technical specifications with which they should comply, are set out in the current document.

The WHO Guidelines for the preparation and establishment of reference materials and reference reagents for biological substances were published in 1978 *(2)*, and were subsequently revised in 1987 *(3)* and 1990 *(4)*. Following scientific and technological advances, the classification of certain substances evolved, with many antibiotics, for example, becoming suitable for characterization using only physicochemical methods. As a result, WHO BRM for many such products were discontinued *(5)*, with only WHO BRM for certain antibiotics now being retained where necessary. At the same time, new WHO BRM increasingly became needed for emerging categories of biological products, such as those developed using molecular biology techniques. These and other developments were subsequently reflected in the WHO Recommendations for the preparation, characterization and establishment of international and other biological reference standards (revised 2004) *(6)*.

Over the past decades, WHO BRM have continued to play a critical role in assuring the quality, safety and efficacy of biological products. However, the underlying concepts, types and applications of WHO BRM have continued to expand and evolve following further technological advances in the development, manufacture and testing of biological products. As a result, a number of important issues have been raised by stakeholders on various occasions regarding the rationale, selection, development, distribution and use of WHO BRM. Specific issues have included the need for global harmonization of terminology, the importance of rapidly responding to public health emergencies, and the need to ensure commutability where relevant. Other issues highlighted have included the complexities associated with stability testing, assay-specific value assignment, and the potential impact on continuity of units following the replacement of a WHO BRM *(7–10)*.

The current document was developed through an extensive consultation process *(11)* to better reflect current practices and to address the above and other issues.

Purpose and scope

This revised WHO Recommendations document provides updated guidance for WHO collaborating centres (WHO CCs), custodian laboratories and other institutions involved in the preparation, characterization, establishment, storage and distribution of WHO BRM. The document also provides guidance for the end users of WHO BRM, for example as part of a manufacturer's overall strategy to ensure product quality and consistency. New and updated elements of the document include:

- consistent use of the term "WHO biological reference material" (WHO BRM) as the umbrella term to denote any biological reference material established by WHO in accordance with the guidance provided in these Recommendations;
- updated descriptions of the three main categories of WHO BRM, namely "WHO International Standard" (IS), "WHO International Reference Reagent" (IRR) and "WHO International Reference Panel" (IRP);
- brief explanation of the role of WHO repositories;
- updated information on the formulation, preparation, optimized processing, quality control, characterization and stability of candidate WHO BRM to be proposed for establishment;
- expanded considerations for the assessment of homogeneity and commutability, and for ensuring the continuity of International Units (IU);
- description of WHO procedures for the endorsement and establishment of WHO BRM, including fast track procedures in response to a public health emergency;
- description of the roles and responsibilities of WHO custodian laboratories;
- guidance on the calibration of secondary standards and other potential uses of WHO BRM, such as the validation or performance monitoring of IVDs; and
- updated information on the standardized nomenclature conventions now used to name WHO BRM.

The document consists of the following main sections and appendices:

- General considerations – sets out the scientific basis of biological standardization, describes the different categories of WHO BRM and outlines the principles of WHO biological standardization.
- Part A – describes the procedures to be followed by WHO and by the laboratories responsible for the preparation, characterization and distribution of WHO BRM.
- Part B – provides considerations on the use of WHO BRM, including for the preparation of secondary standards calibrated against the relevant IS and established by regional or national control authorities, manufacturers or other stakeholders, including diagnostic laboratories. More specific and detailed practical advice and guidance on the preparation of such secondary standards are provided in the corresponding WHO manuals on the preparation of secondary standards for vaccines *(12)*, for IVDs based on nucleic acid or antigen detection *(13)* and for antibody testing *(14)* which should be read in conjunction with the current document.
- Appendix 1 – describes the information to be provided to WHO for the establishment of a WHO BRM.

- Appendix 2 – provides information on the nomenclature conventions to be followed when naming WHO BRM.
- Appendix 3 – outlines the content of the instructions for use (IFU) that accompanies each WHO BRM.

The parts of each section printed in small type are comments or examples intended to provide additional explanation or illustration of the main text.

The provision of digital data standards in the form of reference data sets in addition to physical WHO BRM – for example, to help users further optimize bioinformatics tools and workflows for specific applications (such as the evaluation of high-throughput sequencing methods) is not currently within the scope of this Recommendations document. However, the development, provision and use of such reference data sets is recognized as a topic that may require the development of future specific WHO guidance.

Terminology

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

Accuracy: the closeness of agreement between a measured quantity value and the true quantity value of a measurand.

Analyte: the biological constituent being measured in a biological or other assay.

Assay: a measurement procedure for determining the quantity or presence of analyte(s) based on a model with known principles and a predefined strategy for calculations, including validity criteria and controls.

Binding assay: a measurement procedure based on the interaction between two molecules, namely a ligand (such as a drug, hormone or antibody) and a target (such as a receptor, protein or DNA).

Bioassay (also more formally, **biological assay**): a procedure for the estimation of the nature or biological activity of a material by means of the reaction that follows its application to some elements of a living system (examples include animals, tissues and cells). The biological activity of the material being measured is often defined in **International Units** (or in some cases in International System of Units (SI) units) based on comparison with the reaction of the same system to a biological reference material.

Bulk material: a suitable quantity of the material that will be used to prepare a candidate WHO BRM – bulk material may be obtained from a single source or prepared by pooling material from more than one source, and may be formulated prior to filling into final containers.

Calibration: a value-assignment exercise performed for a secondary standard or an assay.

Calibrator (also elsewhere, **calibrant**): a calibration material used to adjust the output from a measuring system based on, or traceable to, a reference material.

Candidate WHO biological reference material (WHO BRM): the filled and, where applicable, lyophilized material that is subject to evaluation in an international collaborative study.

Commutability: the property of a reference material demonstrated by the closeness of agreement between the relation among the measurement results for a stated quantity in this material (obtained according to two measurement procedures) and the relation obtained among the measurement results for other specified materials.

Continuity: the concept that measurements obtained in terms of the IU defined by a replacement reference material are as similar as possible numerically to measurements obtained in terms of the IU defined by the previous reference material.

Custodian laboratory: the institute or other entity designated by WHO as being responsible for the storage and distribution of WHO BRM.

Dose–response: the relationship between the amount of a material and the assay response it elicits.

Immunoassay (also more formally, **immunological assay**): an analytical procedure that relies on the specificity of an antibody-antigen interaction for the detection and quantification of the target analyte(s).

International Unit (IU): unit assigned by WHO to an IS.

In vitro diagnostic (IVD): a test used for the determination of a physiological or pathological state using samples from the human body.

Matrix: the material in which a measurand is contained.

Measurand: the quantity intended to be measured.

Neutralizing antibody: an antibody that blocks a biological function, thus rendering, for example, a virus non-infectious, a toxin ineffective or an enzyme inactive.

Precision: the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple samplings of the same homogeneous sample under prescribed conditions.

Reference measurement procedure (RMP): a measurement procedure used in the process of providing measurement results for assessing the trueness of values obtained from other measurement procedures for quantities of the same kind.

Secondary standard: a reference material calibrated against an IS.

Similarity: a statistical property of two dose–response curves or lines where they have identical functional forms that differ only in the parameter representing relative activity.

Traceability: the metrological property of the result of a measurement or the value of a material whereby it can be related to stated references (usually national or international standards) through an unbroken chain of comparisons.

Uncertainty: a parameter associated with the result of a measurement that characterizes the dispersion of the values that could reasonably be attributed to the measurand *(15).*

Validation: confirmation, through the provision of objective evidence, that pre-established requirements for a specific intended purpose have been fulfilled.

WHO biological reference material (WHO BRM): any biological reference material established by WHO in accordance with the guidance provided in these Recommendations – for a description of the four different categories of WHO BRM, see "Categories of WHO BRM" in General considerations below.

General considerations

1. Background of biological standardization

WHO BRM are provided for a very broad range of substances, which includes but is not restricted to, proteins, antigens, vaccines, antisera, blood products and nucleic acids. WHO BRM are intended for use in bioassays, immunoassays or other analytical procedures, including those based on biological function (both in vivo and in vitro), immunological reactivity, enzyme activation or amplification, and nucleic acid amplification and detection.

The provision and use of WHO BRM are critically important for maintaining high standards of quality, safety and efficacy for biological products used worldwide for the prevention, diagnosis and treatment of diseases or other conditions. Their use supports the application of many bioassays, immunoassays and other analytical procedures used in the standardization and control of a wide range of often highly complex biological products. Such products include biotherapeutics, cell, tissue and gene therapy products, blood-derived products, and vaccines and other immunological products. Furthermore, the provision of WHO BRM for IVDs not only facilitates the harmonization of clinical sample measurements obtained by different

laboratories, often using different methods, but also supports the validation and performance evaluation of the diagnostic assays used *(16)*.

Assay calibration using WHO BRM is the basis for a common language for test results obtained using different biological, immunological and other assays. This facilitates the common communication of test results and contributes to the harmonization of vital public health activities, such as monitoring immune status, screening for disease or disease susceptibility, diagnosing diseases or other conditions, monitoring therapy outcomes, and testing for blood safety. Successful assay harmonization is also key to reaching international consensus on the interpretation of test results, and for the potential definition of regulatory requirements for the respective tests or products.

WHO BRM are widely used in the development, evaluation, standardization and control of biological products (and related assays) by industry and regulatory authorities, and in biological research in academia and scientific organizations. They thus play a vital role in facilitating the transfer of laboratory science into worldwide clinical practice, and in the development and quality control of safe and effective biological products.

There are a number of special considerations and challenges which apply to the manufacture and quality evaluation of biological products, including the inherent variability of biological systems, bioassays and immunoassays, and the potential for microbial contamination. Historically, the availability of WHO BRM has made a major contribution in addressing these and other challenges, and thus to ensuring the development and use of safe and effective biological products.

The definition of a medicinal substance, used in treatment, prevention or diagnosis, as a "biological" product has been variously based on criteria related to its source, method of production, amenability to characterization by physicochemical means alone, the requirement for bioassays, or on arbitrary systems of definitions applied by regulatory authorities. For the purposes of developing WHO BRM, the list of substances considered to be biological products has historically been related to the definition of "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 increased applicability of chemical synthesis to larger molecules, have made it effectively impossible to base a definition of a biological product on any single criterion related to the structure of the substance, method of production or method of analysis. The establishment of WHO BRM for any substance or class of substances is therefore based on an evaluation of current analytical methodologies, and of the need for higher order standards for these methods, while bearing in mind the different aspects noted above.

> 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 the specific activity of production batches.

In the diagnostics field, the requirement for WHO BRM for otherwise well-characterized proteins and other macromolecules is driven by the routine use of comparative immunological and/or biochemical assay procedures, such as antibody assays and nucleic acid amplification technique (NAT)-based assays, and by the absence of reference measurement procedures (RMPs) to define (often heterogeneous) analytes in absolute terms in complex matrices.

Analogous requirements for WHO BRM exist for measuring the activity or content of diverse measurands, ranging from blood coagulation factors to human genome mutations.

Throughout this document, the term "activity" may refer to biological activity or to other types of activity (such as immunological, biochemical or binding activity) for which a value is assigned to a WHO BRM.

2. Categories of WHO BRM

The main categories of WHO BRM are the International Standard (IS), the International Reference Reagent (IRR) and the International Reference Panel (IRP). WHO BRM containing living organisms are termed "WHO repositories".

2.1 International Standard

The principal category of WHO BRM is the IS. These are substances, classed as "biological" according to the criteria outlined above, that are provided to enable the results of relevant assay procedures to be expressed in the same way throughout the world. The IS, as a primary standard, is considered to be of the highest order and is used for the calibration of secondary standards. IS value assignment is usually in International Units (IU) but another suitable unit may sometimes be used. The IS provides the unique physical basis for the definition of the IU for the relevant activity.

2.2 International Reference Reagent

The category of IRR was established in response to the speed of development of some new biological products *(17)*. A need often exists from both the regulatory and scientific perspectives for reference materials with official WHO status to be available before the clinical utility of the related new biological products becomes apparent. In such cases, the establishment of an IS may not be justified as the material may have only limited use until the clinical utility of the biological product is established.

As an example, the WHO International Reference Reagent for antibodies to Ross River virus for neutralization assays (human, plasma) was established in 2023 *(18)* at a time when no licensed vaccine was available and the utility of Ross River virus antibodies as a potential surrogate marker for efficacy estimation of candidate vaccines was still under investigation.

The availability of this IRR thus enabled assay harmonization and epidemiological studies in advance of specific vaccines becoming used.

A decision to establish an IRR may also be taken in situations where the evidence for fitness for purpose generated from the collaborative study is limited and/or assurance regarding the stability of the candidate WHO BRM is lacking. For IRR that are assigned a value for one or more activities or parameters, this value will be defined as a Unit (U). Such IRR are intended to be interim, with replacement IRR not envisaged. In many cases, sufficient information will have accrued in the period following establishment to allow for the replacement of the IRR with an IS. Only when a material originally established as an IRR is established as an IS will the assigned value be expressed in IU. Where this occurs, it is expected that the assigned IU value will demonstrate continuity with the unitage that was assigned to the IRR following the initial international collaborative study. The assignment of a different value would only be done on the basis of sound scientific reasoning.

As an example, the levels of residual moisture and residual oxygen in individual ampoules of the candidate WHO BRM for antibodies to human papillomavirus type 16 were higher than the usual limits. Therefore, the material was established in 2007 as an IRR with an assigned unitage of 5 U/ampoule and additional stability studies requested *(19)*. Subsequent stability studies indicated no significant change in antibody titre at 14 months of storage at 20 °C of ampoules with either low or high moisture content. On the basis of these results, the IRR was upgraded in 2009 to become the First WHO International Standard for antibodies to human papillomavirus type 16, with an assigned unitage of 5 IU/ampoule *(20)*.

The category of IRR also includes well-characterized, stable materials used as a key reagent in biological or other assays, or to monitor the performance of such assays. These IRR are not used as calibrators and may not have any value assigned to them. When stocks are exhausted, this type of IRR may be replaced by another IRR that will have the same intended purpose.

An example of IRR not intended for use as assay controls and having no assigned value are the WHO international reference reagents for antibodies to human leukocyte antigen *(21)*.

2.3 International Reference Panel

An IRP is a group of related reference materials, often consisting of different variants of the same measurand (for example, different genotypes of a virus) established to collectively aid the evaluation of assays or diagnostic tests. Other IRP consist of members covering a range of different concentrations and/or activities of the same measurand. IRP comply in all respects with the general requirements for WHO BRM set out in this document with the exception that it may not be appropriate to assign an IU to individual panel members following the prior establishment of an IS for the respective measurand. For any defined measurand, there should only be one higher order WHO BRM defining the IU.

2.4 WHO repository

A WHO repository comprises individual preparations of living organisms (for example, well-characterized bacterial strains) for which lyophilization might not be appropriate. Each repository member will be considered for establishment by WHO following the general principles described in this document. Although no units are formally assigned to the individual members of a repository, required information for their use (for example, living bacteria count in colony-forming units (CFU)/mL for bacterial strains) will be determined for each member following a protocol agreed by the WHO Expert Committee on Biological Standardization (ECBS).

> As an example, the First WHO Repository of red blood cell transfusion relevant bacterial reference strains, established in 2019 *(22)* contains five different bacterial strains of known cell count (stored at −80 °C) that are intended for the validation of methods used for the bacterial screening of erythrocyte concentrates under "real life" conditions.

In principle, a similar approach could be followed for other WHO BRM consisting of viable organisms – for example, batches of cells obtained from a well-characterized master cell bank. Other general principles described in this document, including requirements for endorsement, demonstration of fitness for purpose and formal establishment would apply.

2.5 Obsolete WHO BRM categories

During the course of the WHO programme on biological standardization, several categories of WHO BRM have been defined and then later modified or removed, including, for example the materials formerly categorized as "International Reference Preparations".

> For transparency and to avoid any confusion in use or in the literature, WHO BRM that were established in categories that are now obsolete retain their designation and have not been reclassified. However, when a WHO BRM with an obsolete category name is replaced, the current nomenclature should be used. The pathway from the previous category name to the new name should be clearly explained in the IFU accompanying the replacement WHO BRM.

3. Principles of WHO biological standardization

WHO BRM are typically complex biological materials. Activity measured using bioassays and immunoassays often cannot be defined in terms of SI units because the specific biological effect being measured cannot be quantified in terms of physicochemical parameters. As a result, units defined by a suitable reference material are needed for quantifying the biological activity.

During the establishment of WHO BRM, the principles followed are:

- that, where a value is assigned, the assigned value should be in arbitrary units, with any exceptions justified;
- that the unit is defined by a reference material with a physical existence;

- that for the establishment of the reference material, a variety of methods is usually used, and that value assignment (and therefore the definition of the unit) is not necessarily dependant on a specific method of determination;

  Generally, WHO BRM are established for analytes where no internationally accepted reference system (that is, a combination of RMP, reference material and reference laboratory) has been agreed or established. In these cases, the principles set out above will apply. Where an RMP has been defined and agreed, then both establishment and value assignment may be specifically based on that procedure. However, the exercise must be science- and data-driven, and the final value assignment should take into account any significant observations of differences between the methods used.

- that the behaviour of the reference material 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, while it is not necessary for the reference material to be prepared in the same formulation or matrix as test samples, it is necessary that the dose–response characteristics of the reference material demonstrate statistical similarity with those of test samples. For example, the reference material used to assay blood coagulation factor VIII activity in a factor VIII concentrate is derived from factor VIII concentrate rather than plasma.

  However, reference materials may be formulated in such a way as to preserve long-term stability of activity and, where the test systems are not adversely affected, such formulations may differ from the formulation or matrices of the substances to be examined. This means, for example, that the formulation of a blood coagulation factor reference material does not match that of commercial products, that a monovalent vaccine reference material may be used to assay combination vaccine products provided it is shown that the other components of the combination vaccine do not interfere with the response of the vaccine component being assayed, and that the reference material for a diagnostic analyte is not necessarily formulated in plasma or serum despite these being the typical matrices for diagnostic samples.

  For NAT-based determination of DNA or RNA of biological origin (for example, in pathogen detection or oncogene copy analysis) the extraction of nucleic acids (and additionally in the case of RNA, reverse transcription) precedes the amplification step. The efficiency of the nucleic acid extraction system (and, for RNA, of the reverse transcriptase step) may significantly affect the outcome of the analysis. In contrast to the option of well-characterized synthetic nucleic acids, nucleic acid reference materials derived from biological materials undergo the complete procedure used for clinical samples, and are therefore more appropriate for reflecting the combination of different analytical steps involved in NAT-based assays. In accordance with the "like versus like" principle, reference materials similar to clinical samples will therefore be more suitable for standardizing such assays.

  However, in some cases, a reference material that does not qualitatively resemble the test sample could be established provided it has been demonstrated to be suitable

for its intended use. An example of this is provided by the WHO international reference reagents for Ebola virus RNA for NAT-based assays that consist of lentiviral particle-based portions of the Ebola virus genome *(23)*.

The principles described above reflect the challenges associated with standardizing the measurement of complex macromolecular analytes, including:

- difficulties in predicting biological activity based on measurements obtained in SI units;
- the need for measurement in complex matrices (for example, human plasma), the composition of which can vary even between individuals;
- the comparative rather than absolute nature of biological and immunological test procedures;
- the difficulty in quantitatively defining an analyte in terms of a biological response; and
- the difficulty of defining RMPs.

The implications of the above for the establishment of WHO BRM are twofold:

- First, that the measurand is defined by the reference material. This is distinct from the situation for some chemical reference standards which can be fully characterized by physicochemical methods, and for which the measurand is defined by an RMP and/or SI unit.
- Second, it cannot be proven analytically that when an IS is replaced, the IU defined by the new IS will be identical to that defined by the previous one. Therefore, the IU is essentially redefined by the new IS. Where a replacement IS is assigned an activity expressed in IU, every effort is made in the collaborative study design and analysis to maintain continuity of the IU to ensure that the IU defined by the previous and replacement IS are as similar as possible.

  In some cases, the inclusion of a normal plasma pool in the collaborative study may ensure continuity of the IU where appropriate. For example, the IU of blood coagulation factor VIII activity in factor VIII concentrate was established in 1970 and the activity represented by this unit has been maintained through nine successive IS, thus providing a stable baseline over time to assess and compare the efficacy of factor VIII treatments for haemophilia.

Despite such efforts, the IU defined by the first IS and each of its replacements may inherently be different. Furthermore, ensuring continuity of the IU used for the quality control testing of biological products also presents unique challenges due to the use of measurement procedures that may have different selectivities for the measurand, combined with differences in the quality attributes of candidate IS source materials that may be provided by different manufacturers. As the continuity of the IU cannot always be robustly assessed for all users, products or assays during the collaborative study to replace an IS, it is imperative that, for IS used in the quality control of biological products, each user independently assesses the continuity of the IU redefined by a replacement IS using their own analytical procedure and

product. This will allow for the identification of any potential unintended changes in assay results attributable to the replacement of the IS rather than to changes in the product itself.

WHO BRM play an important role in ensuring comparable quality attributes of biological products throughout their life-cycle. Continuity of unit (in the case of replacement IS) and commutability (in the case of WHO BRM used to calibrate diagnostic assays) are key considerations when demonstrating the fitness for purpose of some WHO BRM and are discussed below in more detail in the relevant sections of this document.

4. Value assignment

4.1 Arbitrary units

The basis of the comparative procedures in which many WHO BRM are used is the expression of activity relative to the reference material. Numerous WHO collaborative studies have shown that expressing results as an absolute measurement of biological response – such as a 50% cell culture infectious dose (CCID50) – is more variable than expressing results as a relative activity using a common reference material *(24, 25)*.

For most WHO BRM, the activity is demonstrated by relevant assays and for many IS is stated in IU. The IS thus defines the IU and also has a role in qualitatively defining what is being measured (the measurand). It is implicit that the IU has no existence other than in relation to the IS. For any new WHO BRM, the numerical value assigned will typically be arbitrary, with subsequent replacement WHO BRM value assigned relative to the original WHO BRM. The value assigned to replacement WHO BRM will therefore be dependent on the value assigned to the original WHO BRM and on the combination of methods used to make the comparison.

> For WHO BRM for blood coagulation factors, a normal plasma pool may be used as an additional independent comparator, for example, in studies of candidate replacement WHO BRM.

In some cases, the value assigned to a new (not replacement) WHO BRM may be related to units reported by the assays used for its characterization. For example, the first iteration of a WHO BRM intended for use in NAT-based assays may be assigned a value based on the rounded mean value of NAT-based assay detectable units reported by participating laboratories in the collaborative study.

> As an example, the collaborative study to establish the First WHO International Standard for Zika virus RNA for NAT-based assays resulted in a mean estimate across 70 NAT-based assays of 7.7 $\log_{10}$ (= 50 118 723) NAT-based assay detectable units/mL, and this reference material was therefore assigned a unitage of 50 000 000 IU/mL *(26)*.
>
> For this type of WHO BRM, the logarithmic transformation is now more typically used for unitage assignment, as was the case for example with the First WHO International Standard for

varicella zoster virus DNA for NAT-based assays which was assigned a unitage of 7.0 $\log_{10}$ IU/vial *(27)*.

Rarely, the value assigned to a WHO BRM will be in terms of a "consensus" absolute biological response, but such WHO BRM are not used for the assignment of relative potencies.

An example would be the WHO International Reference Reagent for measles vaccine (live) which was assigned a value in number of infectious units per ampoule but which is used as a control in the assay and not for the expression of relative response *(28)*.

When a replacement IS is established, every effort should be made to assign a value to the new reference material that preserves as closely as possible the value of the IU over time (continuity of the unit). This reduces the risk of users experiencing issues with their assay following the replacement of an IS.

4.2 SI units

Where advances in analytical capabilities, together with a detailed understanding of the measurand, mean that the assignment of a WHO BRM value in SI units may be possible using physicochemical or other suitable methods, then this may be considered. This is particularly relevant in cases where clinical measurements or therapeutic dosing are also based on SI units.

Where applicable, the value assignment of a WHO BRM in SI units should be aligned with already existing regional standards (such as a pharmacopoeial reference standard).

In cases where the value assignment of a WHO BRM is in SI units, the principles of metrological traceability as outlined in ISO 17511 *(29)* should be followed where possible. This will necessitate the existence and use of an appropriate RMP, a certified reference material (CRM) and an assignment of measurement uncertainty. The CRM should be accompanied by documentation issued by an authoritative body and providing one or more specified property values with associated uncertainties and traceability.

However, it may not always be possible to source an appropriate CRM. In such cases, the use of a primary calibrator may be considered, provided the accuracy and metrological traceability of the value assigned to the calibrator can be demonstrated. The composition of a primary calibrator should be assessed on a case-by-case basis, but it will typically possess a much higher content of the measurand than the proposed WHO BRM, and lack excipients, to allow for the accurate assignment of its content in SI units using an appropriate primary RMP.

A primary RMP is a measurement method that is fully described, validated and metrologically traceable to the SI to provide the highest achievable accuracy for a given measurand. Such RMPs include isotope dilution mass spectrometry for proteins, quantitative nuclear magnetic resonance spectroscopy for carbohydrates and peptides/small proteins, and digital polymerase chain reaction (PCR) for purified DNA. The primary RMP forms the basis for assigning SI-traceable values to the CRM and, where applicable, to the WHO BRM. The development of a

suitable RMP requires sufficient knowledge and documentation of the factors affecting the outcome of a measurement and the uncertainty associated with measurement results. Most biological assays are therefore not suitable for use as RMPs since the factors that affect the results of such assays cannot be fully described. The value-assigned CRM or primary calibrator can then be used to calibrate the proposed WHO BRM using a method more amenable to the reduced content (and matrix) of the proposed WHO BRM, such as high-performance liquid chromatography combined with mass spectrometry (HPLC-MS) or an ultraviolet detector (HPLC-UV). As a rule, where SI units are assigned to a WHO BRM they should be derived from validated procedures with acceptable performance characteristics.

> An example is the First WHO International Standard for thrombin activatable fibrinolysis inhibitor (plasma), which is intended to harmonize the measurement of thrombin activatable fibrinolysis inhibitor (TAFI) activity and antigen in human plasma and has values assigned in IU for both. For consistency with the many commercially available calibrators for TAFI antigen with assigned values in SI units (g/mL), a TAFI antigen content of 7.43 µg/ampoule (expanded uncertainty = 7.05–7.82 µg/ampoule) was also assigned to the IS. This value was assigned via an isotope dilution mass spectrometry method relative to a primary calibrator consisting of purified TAFI protein that had been value assigned by amino acid analysis *(30)*.

> A further example is provided by the Second WHO International Standard for insulin-like growth factor 1 (recombinant, human) which is intended to harmonize immunoassays used for the measurement of insulin-like growth factor 1 (IGF-1) in human serum. An overall geometric mean IGF-1 content of 33.0 µg/ampoule (expanded uncertainty = 30.5–35.6 µg/ampoule) was determined using 23 valid HPLC assays against a primary calibrator with a previously assigned mass value of 1.045 mg/vial based on amino acid analysis *(31)*.

There are also a small number of IS which have been value assigned in SI units despite the absence of both an RMP and CRM, or alternative in-house primary calibrator with SI unit traceability. This approach may be justified where clinical decisions are made on the basis of well-established SI values (such as mg/L or mol/L) *(29)*. The collaborative study report should be consulted for full details regarding the metrological traceability of the assigned value.

> An example is the Second WHO International Standard for prostate specific antigen (free) with an assigned content of 0.53 µg/ampoule despite the absence of full traceability for the SI unit assigned. In this case, value assignment was based on the use of an established and widely used commercial assay *(32)*.

4.3 Assignment of more than one value to the same WHO BRM

It is recognized that many biological materials (and therefore many WHO BRM) exhibit more than one biological or other activity and thus, where appropriate, the same WHO BRM may be used to measure one or more of these activities.

> Examples of assigning values for different biological activities to the same WHO BRM include: (a) the Third WHO International Standard for low molecular weight heparin, established in 2012 *(33)* which was assigned values in IU for anti-Xa activity and anti-IIa activity; (b) the

Third WHO International Standard for protein S (plasma), established in 2023 *(34)* which was assigned values in IU for functional activity, free protein S antigen and total protein S antigen; and (c) the First WHO International Standard for rituximab, established in 2017 *(35)* which was assigned values in IU for complement-dependent cytotoxic activity, antibody-dependent cell mediated cytotoxic activity, cell-binding activity and apoptotic activity.

Another example is provided by WHO BRM for the measurement of antibody responses in infectious disease serology. Such reference materials may exhibit both antigen-binding and pathogen (or toxin) neutralization activities and be useful for different purposes (for example, the harmonization of binding assays and neutralization assays respectively). While it may be possible to establish a single WHO BRM with values assigned for more than one activity/purpose, it has been recognized that this approach may create the false impression that binding activity IU and neutralizing activity IU are equivalent before sufficient evidence has been obtained to demonstrate this. As a result, some WHO BRM of this type are established as separate WHO BRM from the same material, each of which should be labelled accordingly.

Examples include the First WHO International Standard for antibodies to Rift Valley fever virus for neutralization assays (human plasma) and the First WHO International Standard for antibodies to Rift Valley fever virus for binding assays (human plasma) *(36)*.

In all cases, the approach taken will depend on the nature of the WHO BRM and on the assay systems in which it is intended to be used.

4.4 Other or no values assigned to WHO BRM

Rather than being assigned a value in IU or SI units, some WHO BRM are assigned a value in terms of a relevant property.

Examples include: (a) the First WHO International Reference Panel for genomic KRAS codons 12 and 13 mutations established in 2017, with assigned consensus mutation percentage values and consensus mutant and total KRAS copy numbers *(37)*; and (b) the Fifth WHO International Standard for thromboplastin (recombinant, human, plain) with an assigned value based on the International Sensitivity Index *(38)*.

In some cases, a WHO BRM intended to be used, for example, for qualitative purposes is established with no assigned value.

Examples include: (a) the WHO IRR for RBC1, RBC4, RBC5, RBC12 blood group genotyping alleles (genomic DNA) established in 2011 to check the accuracy of genotyping assays *(39)*; and (b) the WHO IRR for gut microbiome analysis established in 2022 to check the proficiency of high-throughput sequencing technologies *(40)*.

4.5 Metrological aspects

The extent to which measurement uncertainty, as defined in the standard ISO 17511 *(29)*, applies to WHO BRM has been raised. particularly in regard to IVDs. However, because an IS defines the IU, the IU has no existence other than in relation to the IS that defines it and

therefore an uncertainty value is not given. This principle also applies to replacement IS (where the replacement will define the IU) and to the small number of IS that are assigned SI units despite a lack of metrological traceability due to the absence of both an RMP and CRM or SI-traceable in-house primary calibrator *(32)*.

Another issue raised by ISO17511 *(29)* 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 materials regardless of any other considerations. Following ECBS consideration of this issue, it has been concluded that the choice of unit should reflect, and be based on, the intended use as well as the physicochemical information available on a case-by-case basis.

> According to ISO 17511 *(29)* many IS would be considered to be "international conventional calibrators" characterized by the absence of both an SI-traceable RMP and certified SI-traceable reference materials.

5. Measurand

The quantity intended to be measured by an assay is defined as the measurand. In most cases, the definition of the measurand will be reflected in the procedures used to characterize and assign a value to the WHO BRM. Thus, WHO BRM intended for the calibration of bioassays will generally be characterized using bioassay procedures, those for immunoassays using immunoassay procedures, and so on.

In some cases, and in particular where the reference material is sufficiently well defined to allow for complete characterization using physicochemical methods, determination of the measurand will be achieved using an RMP distinct from the routine assay procedures. This approach is comparable to that used in clinical chemistry for analytes which, while routinely assayed by immunoassays, may be measured as defined molecular entities by spectroscopic or other methods for the purpose of assigning a quantity value.

- Examples include: (a) the Second WHO International Standard for somatropin (recombinant DNA-derived growth hormone) used as a primary calibrator for clinical immunoassays for growth hormone, and assigned a value in mg, traceable via amino acid analysis of a physicochemically defined preparation; and (b) synthetic DNA preparations used in the calibration of the amplification step of NAT-based assays (such as PCR) and assigned a value based on phosphate or nucleotide determination or single molecule enumeration.

6. Commutability

Commutability is the property of a reference material that relates to the degree of agreement between the results obtained for that reference material and those obtained for representative samples of the type intended to be measured when assessed in two or more measurement

procedures. Such agreement is important when the intended use of the reference material is to harmonize the results obtained for samples using different measurement procedures.

Commutability is therefore of vital importance for reference materials intended for use in the measurement of clinical samples in laboratory medicine. Measurement procedures that are calibrated with commutable reference materials will produce results for clinical samples that are equivalent across different procedures, providing assurance that the results obtained and clinical decisions made will be consistent regardless of the method or platform used. The different procedures used should all have the same selectivity for the measurand. Selectivity is a relative term to describe the extent to which particular analytes in mixtures or matrices can be measured without interference from other components with similar behaviour. Where procedures have a different selectivity, equivalent results may not be obtained even if the reference material is commutable. Conversely, a non-commutable reference material is unlikely to harmonize results across measurement procedures, even those with the same selectivity for the measurand, and could lead to discrepancies in test outcomes, resulting in incorrect diagnoses and/or ineffective treatment decisions. Potential differences between the reference material and clinical samples with respect to composition (for example, in the molecular form of the measurand, matrix and/or excipients) and/or processing (for example, lyophilization) may cause non-commutability. However, the reasons for non-commutability are not predictable and non-commutability may only be observed with some, but not all, measurement procedures. Where non-commutability is observed, it can be difficult to distinguish composition-related causes from influences related to the non-selectivity of the measurement procedure(s) for the measurand in question. For this reason, measurement procedures to be included in a commutability assessment must have adequate selectivity for the measurand. In addition, a reference material containing several different measurands might be commutable for some of the measurands but not for the others, and commutability should therefore be assessed independently for each measurand.

The assessment of commutability for a reference material with a diagnostic application is a crucial but complex undertaking, ideally involving all of the commonly used measurement procedures and relatively large numbers of representative clinical samples. Any project to produce a reference material for IVD use should factor in the critical need to demonstrate commutability when planning the collaborative study to establish a WHO BRM.

Assessment of commutability for other types of WHO BRM (that is, those not intended for diagnostic use) may be desirable, but in many cases may not be feasible. For example, a thorough assessment of the commutability of an intended WHO BRM for vaccine biological activity would require the inclusion of representative batches of the vaccine from as many producers of that vaccine as possible. Given that many of the assays used for measuring vaccine biological activity are not high-throughput assays (for example, cell-based biological activity assays and in vivo biological activity assays), the resource demands on participating laboratories to test all of the products would be prohibitive.

However, it should also be noted that the concept of commutability in its application to clinical diagnostics (where the ultimate aim is that the result obtained for a given patient sample is the same regardless of the laboratory performing the assay or the measurement procedure used) does not necessarily extend to other biological measurements and samples.

For a typical vaccine product, any batch of that product will be tested in a relatively small number of laboratories (for example, the manufacturer's laboratory and one or more control laboratories) with, in most cases, regulatory decisions being based on product-specific acceptance criteria. The commutability of a WHO BRM for a vaccine antigen could be assessed on a product-specific basis among small groups of laboratories (for example, the product manufacturer and national control laboratories involved in testing that product) but this would only be possible following establishment of the WHO BRM and not as part of the original collaborative study to assess the suitability of the candidate WHO BRM.

In some collaborative studies for candidate WHO BRM not intended for use in diagnostic assays, one or more representative "test" samples of the type intended to be measured may be included and tested by all study participants to generate data that could be used to make a limited assessment of commutability. Although this information is valuable, any conclusions that could be drawn would be restricted to the type of "test" samples included, which may represent only a small proportion of all sample types available worldwide from producers of that product type.

Part A. Recommendations for the preparation, characterization and establishment of WHO biological reference materials

The recommendations provided below are intended to reflect best established practice for the preparation, characterization and establishment of WHO BRM. These recommendations therefore serve as guidance for any laboratory or organization involved in preparing and testing candidate reference materials intended for such a purpose.

Whenever animals are used to produce or characterize a WHO BRM, their use and husbandry should be subject to regulatory oversight, and should adhere to strict ethical standards in accordance with national and international conventions on the use and welfare of animals in research.

A.1 Overview of WHO BRM procedures

A.1.1 WHO collaborating centres and WHO custodian laboratories

WHO designates certain institutions as WHO collaborating centres (WHO CCs) to carry out activities in support of its programmes of work. A number of WHO CCs support the WHO biological standardization programme by identifying needs for new WHO BRM, obtaining or helping to obtain source materials, and contributing to the characterization of candidate WHO BRM. Some WHO CCs are also designated as WHO custodian laboratories with responsibility

for storing and distributing WHO BRM. The custodianship of WHO BRM requires considerable commitment and investment by the host institution. A WHO custodian laboratory will typically lead and manage the project to produce and characterize a new or replacement WHO BRM, sometimes in close collaboration with one or more WHO CCs. To help identify public health needs and/or develop required new WHO BRM, WHO custodian laboratories may also work in close collaboration with other scientific organizations (for example, the International Society on Thrombosis and Haemostasis), other WHO programmes (for example, IVD Prequalification) and other stakeholders (for example, providers of external quality assessment programmes).

WHO CCs may also belong to thematic networks for discussing and aligning standardization activities, maximizing the use of resources and avoiding duplication of effort. Examples of such networks include the WHO network of collaborating centres on standardization and regulatory evaluation of vaccines, and the WHO network of collaborating centres for blood products and in vitro diagnostics.

A.1.2 Other standardization organizations

Expansion of the scope of work undertaken in the field of biological standardization has led to a number of other laboratories and organizations becoming involved in the production of materials that may ultimately be offered to WHO for potential establishment as WHO BRM. For this reason, the ECBS has requested that WHO foster cooperation and the sharing of information among the various relevant international associations, scientific bodies and other stakeholders to avoid duplication of effort *(41)*, with close cooperation with a WHO custodian laboratory encouraged. In some circumstances, WHO may establish collaborative links with other standardization organizations to jointly pursue specific standardization projects that were prioritized and initiated independently.

A.1.3 Assignment of priorities for the development of WHO BRM

A proposed replacement WHO BRM is assumed to already have a demonstrated global need and public health importance. To ensure continuity of supply, the development of such WHO BRM will normally be given the highest priority *(42)*. Prioritization considerations for the development of new WHO BRM include:

- the public health importance of, and global need for, the new WHO BRM from a regulatory and scientific perspective;
- the approval status of the relevant biological product or IVD; and
- the number of products that have been approved, or are in development, or the number of different diagnostic assays in the case of IVDs.

Higher priority will normally be given to WHO BRM intended to be used for approved products or established diagnostic assays, compared to those for which products (or assays) are still in the development stage.

The development of WHO BRM of global importance and use will typically be given higher priority compared to those of regional importance and use.

In some cases (for example, to support the response of WHO to a public health emergency), a higher priority will be given to the development of a WHO BRM for which biological products or diagnostic assays may not yet be approved, or that have been approved under emergency use authorization only. This may include WHO BRM for use in public health emergencies that are likely to be restricted to certain regions but where the public health impact is high.

A.1.4 Endorsement and establishment of WHO BRM

Any proposal to develop a new or replacement WHO BRM, or to change the status of an IRR to an IS (see also "Categories of WHO BRM" in General considerations above) must be endorsed by the ECBS. Such proposals are typically submitted by custodian laboratories or, occasionally, by other WHO CCs or stakeholders working in cooperation with a custodian laboratory. Proposals must include an explanation of why the reference material is needed, together with information on its anticipated uses and users. Information on the source of the candidate WHO BRM is also to be provided, along with the proposed design of the collaborative study and any issues that need to be brought to the attention of the ECBS. Proposals should also include considerations relevant for prioritization (see section A.1.3 above). The decision of the ECBS with regard to endorsement is recorded in the formal report of the meeting which will then be published in the WHO Technical Report Series. Rarely, an endorsed project may subsequently not proceed – for example, due to a change in the epidemiological situation, advances in technology negating the need for the WHO BRM or an inability to obtain suitable source material. In such cases, a justification must be made to WHO explaining why the project should not continue. As with the original endorsement, any recommendation from the ECBS not to continue with an already endorsed project is recorded in the meeting report and published in the WHO Technical Report Series.

Following completion of the collaborative study to evaluate the candidate WHO BRM, the coordinating laboratory will prepare an initial summary report. Prior to its submission to WHO, the summary report should be sent to each collaborative study participant and comments invited with regard to the conclusions drawn and recommendations made. Following submission to WHO, the summary report will be assigned a "Biological Standardization" (BS) number by WHO and posted on its website for public consultation. The BS summary report then serves as the basis of the respective presentation to the ECBS. The information to be included in the BS summary report in support of the establishment of a WHO BRM is described in more detail in Appendix 1 below.

The candidate WHO BRM may then be established as a WHO BRM provided that:

- the BS summary report has been prepared with all study participants having had the opportunity to comment on its contents, and the report (including all participant comments received) has been submitted to the ECBS;

- the public consultation of the BS summary report undertaken by WHO prior to its presentation to the ECBS has been completed;
- any queries raised by working groups or other entities requested by WHO to provide peer review of the proposal(s) have been answered satisfactorily;
- any queries raised by members of the WHO Expert Advisory Panel on Biological Standardization following consideration of the information provided in the BS summary report have been answered satisfactorily;
- a summary of the project and its outcomes, including all aspects raised in the public consultation, has been presented to the ECBS; and
- the ECBS reaches a consensus for establishment based on the evidence provided.

The ECBS will then make its recommendation to WHO regarding the establishment or otherwise of the WHO BRM, including assigned unitage where appropriate. Based on the comments received from the public consultation, and on the meeting discussions, the ECBS may request that some modifications be made to the BS summary report. The final version of the document will then be posted on the WHO website. For each proposed WHO BRM, the project outcomes, the main discussion points raised and the recommendation of the ECBS will be summarized in the ECBS meeting report published in the WHO Technical Report Series. All final decisions regarding the establishment of a WHO BRM will be made by WHO.

A.1.5 Fast-track procedure

To support a WHO response to a public health emergency, some WHO BRM may be established under a fast-track procedure. The general principles described in this document still apply and should be followed but due to the urgent need for a WHO BRM to be made available, WHO may agree to its establishment based on reduced collaborative study data at the time of submission to the ECBS, providing the fitness for purpose of the candidate WHO BRM has been satisfactorily demonstrated – for example, by a collaborative study involving a smaller number of laboratories than would normally be expected. WHO may also organize ad hoc ECBS meetings to facilitate the fast-track endorsement and establishment of such reference materials. Additional characterization data obtained post-establishment should be submitted to WHO and the ECBS when available.

> In response to the COVID-19 pandemic, WHO organized more frequent ECBS meetings during 2020 *(43, 44)* to facilitate the expedited endorsement and establishment of IS – for example, for SARS-CoV-2 neutralizing antibodies, SARS-CoV-2 RNA and SARS-CoV-2 antigen – as part of efforts to ensure prompt access to the well-characterized reference materials urgently needed to harmonize assay results reporting and thereby allow for the meaningful comparison of early study findings.

A.1.6 Discontinuation or withdrawal of WHO BRM

Some WHO BRM may be formally discontinued by WHO. Any proposal to discontinue an existing WHO BRM should follow the same broad outline of the procedure described above for endorsement. Discontinuation is rare, and the reasons for it may vary depending on the specific situation. For example, a proposal to discontinue an existing WHO BRM may be submitted if the reference material no longer reflects the state of the art or has very restricted

geographical utility. The public consultation that takes place as part of this process provides an important opportunity to obtain feedback from stakeholders and to ensure that no critical gaps are introduced into the control of required biological products or diagnostics for public health. Custodian laboratories should also contact users of the WHO BRM to obtain feedback on the proposed discontinuation. Discontinued WHO BRM are likely to remain available from the custodian laboratory until stocks are exhausted, but a replacement WHO BRM will not be established by WHO following endorsement of a proposal to discontinue its use.

In very rare cases, a WHO BRM may need to be withdrawn (for example, after evidence emerges that it is no longer fit for purpose). In such cases, every effort should be made by the custodian laboratory to develop and propose a suitable candidate WHO BRM for establishment as a new WHO BRM.

Any recommendation made by the ECBS for the discontinuation or withdrawal of a WHO BRM will appear in the ECBS meeting report published in the WHO Technical Report Series, and will be reflected through the updating of the WHO BRM catalogue (see section A.1.7 below).

A.1.7 WHO BRM catalogue

WHO holds and updates a catalogue of all currently available WHO BRM on its website.[1] The catalogue includes information on the year of establishment of each WHO BRM, the responsible custodian laboratory and the relevant BS summary report. The catalogue is regularly updated to reflect the recommendations made by the ECBS with regard to the establishment or discontinuation of WHO BRM. WHO custodian laboratories also provide online catalogues that include all of the WHO BRM for which they are the custodians.

A.2 Safety considerations

Many biological materials, including those of human or animal origin, intended for the preparation or characterization of a WHO BRM can be considered to be potentially hazardous. For reasons of safety in handling and use, materials must be obtained appropriately and screened for the presence of specific infectious markers where applicable. Blood should be obtained from donors who meet current international requirements *(45)*.

> Screening of human blood/plasma donations will involve the testing currently required in many countries for human blood and plasma, namely NAT-based assays for the bloodborne viruses HBV, HCV, HIV-1 and HIV-2, and other relevant pathogens, in addition to antibody testing *(45)*.

The actual or potential infectivity of biological materials of human or non-human origin, especially those derived from virus cell cultures or bacterial cultures, should be taken into

[1] https://www.who.int/teams/health-product-policy-and-standards/standards-and-specifications/norms-and-standards/catalogue

account. The epidemiological situation regarding infectious agents in the region of origin of source materials (such as human plasma or serum) needs to be considered. Suitable procedures may be applied to inactivate microorganisms or their components and the effectiveness of such inactivation should be demonstrated.

> For example, the human plasma donations used to produce the First WHO International Standard for Ebola virus antibodies (plasma, human), established in 2017, had undergone solvent detergent inactivation prior to the lyophilization step, with no residual cell culture infectivity measurable *(46)*.

The impact of any inactivation process on the fitness for purpose of a candidate WHO BRM should be investigated, including any potential interference of inactivation with the measurement of the respective analyte(s) in the intended assays. Such investigations may include the parallel testing of inactivated materials and their native sources in the relevant assays.

It is essential that suitable precautions are taken in the user laboratories when handling and disposing of biological materials to avoid possible infection. This is particularly important when the material is known to be infectious.

Furthermore, it is essential that appropriate precautions are taken to ensure that shipments of materials, including source materials, study samples, and the WHO BRM once it has been established, comply with international regulations on the transport of infectious substances. A number of considerations regarding the communication of relevant safety information to the recipients of such materials are described in section A.10.3 below.

A.3 Quality assurance

A.3.1 Quality management system

WHO BRM should be produced, stored and dispatched under a defined quality management system – for example, ISO 9001 for overarching quality management *(47)*, ISO 17034 for the competence of reference material producers *(48)*, ISO 13485 for medical devices *(49)*, ISO 15194 for reference materials for IVDs *(50)*, ISO 17511 for metrological traceability of assigned values *(29)*, ISO/IEC 17025 for testing and calibration laboratories *(51)* and ISO 15189 for medical laboratories *(52)*. It is desirable that the quality management system(s) in place are assessed as satisfactory by an independent body.

Other essential aspects of the preparation and characterization of WHO BRM may be partly, or entirely, outside the control of the coordinating laboratory. These include:

- the preparation and characterization of source materials in provider laboratories;
- the characterization of materials and trial formulations in testing laboratories;
- the contribution of the ECBS and other consultative committees to the collaborative study design; and

- the testing performance of collaborative study participants.

Although such activities may be conducted outside the control of the coordinating laboratory, it is strongly recommended that processes and documentation compliant with recognized quality standards are, to the fullest extent possible, implemented and followed.

A.3.2 Records

In compliance with quality system requirements, it is essential that complete records are kept that encompass, but are not restricted to:

- the background and proposals for the preparation of the intended WHO BRM;
- the responsible persons and their defined roles;
- certificates of analysis of bulk materials intended for use in the preparation of the candidate WHO BRM – if materials are of human origin (for example, human serum or plasma) information on individual donors and on the epidemiological background of the donor population should be available;

  Information on donors may include details of the donation centre, the sex and age of donors, potential health conditions or disease, and records of ethical approval for the donations and of the obtaining of informed donor consent.

- the procedures and tests performed before, during and after filling into containers, including quality control tests for residual moisture (for lyophilized reference materials), oxygen headspace and homogeneity (homogeneity tests may also include biological and other assays determining the measurand) – the results and raw data for all tests performed should be recorded;
- the stability studies conducted and their outcomes;
- raw data from the collaborative study;
- the summary report of the collaborative study, including related recommendations; and
- records of the agreement or otherwise of study participants with the contents of the summary report.

Such records form the basis of the establishment of the WHO BRM and, where applicable, of value assignment. They should therefore be retained even after a WHO BRM is replaced and should be kept until such time as the WHO BRM is discontinued and no longer replaced.

In addition, records relating to storage, inventory and dispatch of the WHO BRM should be kept, as well as records of all user feedback and experiences regarding its use.

A.3.3 Qualification and maintenance of equipment

The quality system should clearly identify critical equipment and technology, along with procedures for their qualification and maintenance to ensure their functionality. Such critical equipment includes, but is not restricted to:

- analytical equipment for determination of freezing and critical transition temperatures;

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

A.4 Nature, source and storage of bulk material

A fundamental principle of WHO BRM suitability for use is that the behaviour of the reference material should resemble as closely as possible the behaviour of test samples in the assay systems used to test them. To reflect this principle, a candidate WHO BRM should demonstrate statistical similarity with relevant test samples in the assay systems used.

The bulk material selected should have an activity level sufficient for the purposes of the assays or tests for which it is to be used. Although the candidate WHO BRM does not necessarily have to be of the highest purity, no other substances present should interfere with any of the procedures in which it is intended to be used (see also "Commutability" in General considerations above).

> Generally speaking, the nature of the candidate WHO BRM 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 activity, its physicochemical characterization and absence of significant contaminants. Plasma products will reflect current manufacturing technologies, and in addition will be provided with certificates demonstrating compliance with current safety and ethical requirements. Vaccine reference materials will reflect current practices in the preparation of microbial immunogens.

The bulk material containing the measurand will usually be obtained from a single source. It may consist of part or all of a single batch. More than one bulk material may be obtained to enable comparison of two or more candidate WHO BRM. Comparing more than one candidate WHO BRM is often more relevant for the establishment of the first WHO BRM for a given measurand compared to the establishment of replacement WHO BRM for which practical experience in assessing the relative suitability of different materials may already have been gained.

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

If it is necessary to prepare the bulk material by pooling material from more than one batch or source, as will typically be the case for candidate WHO BRM prepared from human serum or

plasma, the procedure employed should ensure that the pooled material is mixed thoroughly and is homogenous. For bulk liquids containing proteins or other labile components, care should be taken to avoid denaturation or degradation during mixing. In addition to any studies that may have been conducted on the individual batches before pooling, the suitability of the homogeneous blend should be demonstrated.

For some candidate WHO BRM, the source material may need to be diluted (or spiked) into a relevant matrix – for example, a virus isolate or purified protein may be diluted in human serum or plasma to achieve the desired concentration, and to ensure that the eventual WHO BRM will resemble as closely as possible the samples with which it is intended to be used. Where this is done, the matrix/diluent should be evaluated to confirm that it does not contain substances that may impact on the performance of the candidate WHO BRM.

> For example, the Third WHO International Standard for hepatitis B surface antigen was prepared using a concentrated and purified antigen bulk that was diluted in thrombinized and declotted human plasma. The human plasma was tested and shown to be negative for anti-HBs since the presence of specific antibodies in the plasma could have reduced the amount of hepatitis B virus surface antigen available for detection, potentially rendering the candidate WHO BRM unfit for purpose *(53)*.

When the bulk material used to prepare a candidate WHO BRM is of commercial origin, this fact should not be used by the provider for advertising or marketing purposes.

In order to serve as a WHO BRM, a sufficient number of final filled containers (preferably enough to last at least 10 years) should be available to meet the estimated demand and to avoid the need for frequent replacement. This requirement should be considered when deciding upon the quantities of source material to be obtained. As the amount of bulk material needed will depend on estimated demand, smaller batch sizes may be suitable in cases where only a limited number of laboratories are expected to request the WHO BRM.

When source materials for a candidate IS are being obtained, the possible approaches to be taken for the eventual replacement of the IS should be considered, particularly in relation to continuity of units. Such approaches may include:

- obtaining and holding excess bulk materials to allow for replacement with an identical material (subject to satisfactory stability);
- including multiple potential future replacement IS in the collaborative study in addition to the candidate IS (subject to satisfactory stability); and
- where long-term stability can be assured, extending the lifetime of the IS by preparing larger fills (up to 20 000 containers), and/or encouraging the establishment of regional or national secondary standards to reduce the rate of use of the IS (see Part B below).

The bulk material must be stored under suitable conditions before being further processed and distributed into final containers. Recommended suitable storage conditions should be provided

by the provider of the bulk material. Where possible, a smaller aliquot of the bulk material should be provided in a separate container to enable preliminary characterization and/or development work to be performed (see section A.5.2 below) without the need to sample from the main bulk. This can help minimize any contamination risk and avoid the freeze–thawing of bulk materials that are stored frozen. Where this is not possible, sufficient volume to allow all necessary testing should be removed from the bulk before it is placed in storage. The sample should be stored under the same recommended storage conditions as the bulk.

In all cases, the storage conditions should ensure that the activity and quality/integrity of the material is conserved.

Some biological materials may be inherently labile and may have a short shelf-life or hold time before they must be processed and lyophilized. Such materials should be identified in advance (in discussion with the material provider) and careful planning of the timelines for receipt and further processing of the material will need to be undertaken by the responsible laboratory.

A.5 Design and preparation of candidate WHO BRM

A.5.1 Containers

The majority of WHO BRM are provided in lyophilized form in sealed glass ampoules. 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 *(54)*. Vials with suitably dried stoppers can be used as an alternative to glass ampoules to provide comparable moisture levels *(55)*. In all cases, the chosen container and closure should ensure the long-term stability of the proposed WHO BRM, with small-scale pilot studies being performed, if required, to inform the choice of container for the final product.

For certain types of biological material, such as infectious materials, stoppered vials are recommended. This may also be the container of choice for inactivated products that are handled in a high-containment laboratory alongside clinical samples, as the stoppered vials can be opened without breaking the glass container, therefore reducing the risk of infection arising from broken glass puncturing the skin when handling the clinical samples. The use of stoppered vials also allows for reconstitution of the lyophilized material by injection of diluent through the rubber stopper, mitigating the risk of potential inhalation exposure.

Containers should, as a minimum, be made of neutral (borosilicate) glass type I of appropriate quality – for example, complying with the current requirements of the European Pharmacopoeia or U.S. Pharmacopeia. The glass must be free from stresses and the containers must be able to withstand sterilization by heat and other temperature stresses, such as those resulting from rapid freezing to −80 °C. Optional sterilization techniques (e-beam, gamma irradiation or ethylene oxide) may be used, especially in the case of pre-sterilized “ready-to-use” containers. Actinic (brown) glass may be necessary for photosensitive materials but does not allow the contents to be seen as clearly. If stoppered vials are used, the closures should be

of appropriate quality – for example, complying with current pharmacopoeial requirements for closures for injections.

A specification for the purchase of containers (and, if necessary, of closures) should be established. All batches intended for use should be shown to conform to the specification. Where ampoules are used, their shape and size should be such that they can be easily filled, 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 containers for materials to be lyophilized since this ensures good thermal conductivity between the bottom of the ampoule and the surface of the shelf in the freeze dryer.

The containers should be cleaned using a process that does not involve the use of a detergent. Sterilization via heat is recommended for the decontamination of all glassware and consumables used in the production process. The chosen method should have a full validation history and consideration should be given to the use of additional heat cycles that may be required to remove ubiquitous contaminants that may negatively impact upon the fitness for purpose of the final product. If the clean containers are to be stored at any point before filling, they should be placed in sealed dust-proof containers. Another option is to use ready-to-use containers from commercial suppliers, which arrive in sealed packages and have already undergone cleaning and sterilization.

The size (volume) of the containers used will depend on the amount of material required in each container, with 5 mL containers generally being used for 1 mL fills. Containers with a capacity of 2.5 mL have also been used for smaller fill volumes of 0.25–1.00 mL.

A.5.2 Formulation and process optimization

Candidate WHO BRM should be prepared using conditions and formulations for which it has been demonstrated that the activity and/or other relevant properties of the material are not degraded or lost, that the activity of the final material is stable and that the finished product is suitable for its intended use. Where the candidate WHO BRM is a replacement, much of this information will be available. However, candidate new WHO BRM will require research and development to determine suitable conditions and formulations. This is achieved by carrying out and analysing small-scale trial fills under conditions that mimic as closely as possible the conditions to be used for the large-scale definitive fill. A research and development programme should be clearly defined and recorded. Formulation conditions that optimize the retention of activity upon freeze drying will assist in the development of new WHO BRM *(56)*.

The scientific understanding of freeze drying has progressed significantly over time *(57–59)*. However, while such improved knowledge may increase the likelihood of preparing a suitable production batch at the first attempt, the source material used to develop a candidate WHO BRM may be unique, or extremely difficult to source. Formulation and process development

work is therefore strongly encouraged to reduce the risk of source material loss, especially for new reference materials.

The choice of process and the extent of processing required to prepare the final bulk for filling will depend not only on the nature and concentration of the active ingredient and its matrix, but also on whether the liquid bulk is a true solution, a colloid or a suspension. In all cases, 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 (for example, by filtration) to control microbial contamination or to remove particles or aggregates of active material. Candidate WHO BRM based on plasma or serum are examples of where filtration may be required. 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 be pyrogen free, where appropriate.

Where the inclusion of an antimicrobial preservative is necessary, it should be one that will not adversely affect the suitability for use of the candidate WHO BRM, volatilize during the drying process or decrease the stability of the material.

The choice of preservative is an important consideration as some countries place restrictions on which preservatives are acceptable. The choice of preservative should be justified, and records of this should be retained. Due to their toxicity profiles, cresol or phenol should not be used if feasible. Sodium azide (which may form explosive compounds with metals) should not be used as a preservative in candidate WHO BRM that are to be freeze-dried.

The analyte is frequently present in a container in such small amounts that a bulking agent will be used in the filling solution to allow a visible freeze-dried plug of suitable size to be formed. In some cases, materials are added to prevent or limit adsorption of the analyte onto to the internal glass wall of the container, and/or to prevent structural changes affecting activity that may occur during freeze drying. Any added substance should not have adverse effects on the activity of the material, or interfere with the assays or tests for which the candidate WHO BRM is intended.

> If a protein stabilizer derived from human blood (such as human albumin) is used, it should comply with current requirements for blood products regarding freedom from contamination *(45, 60)*, and proteolytic enzymes should be minimal. If a protein derived from animal sources is used, consideration must be given to the relevant import/export regulations. Some countries may require a recipient to obtain permits to import the final goods. These permits may demand that the shipper/manufacturer provides an “export health certificate” that testifies to the health of the donor animal. This certificate is often required to be issued by national authorities of the exporting country and signed by a veterinary professional. This can be complex if the manufacturing country differs from the donor animal country of origin, or where there is a chain of suppliers. Therefore, documentation attesting to the source and health status of donor animals should be obtained at the point of material donation to avoid subsequent complications with

regard to regulatory requirements. The use of certain sugars as bulking agents, particularly those with reducing groups (such as lactose), should be avoided as they can form stable complexes with free amino groups in proteins.

Preliminary freeze-drying trials involving extensive comparative analysis of the dried material and liquid bulk may be necessary to establish that freeze drying itself (or an added substance in the formulation) has not significantly affected the activity and fitness for purpose of the candidate WHO BRM. Such studies should also include investigation of the stability of the reconstituted trial material. A small reduction in activity following lyophilization is not unexpected for many biological materials but, provided that activity remains sufficiently high for the intended use, and the stability of the lyophilized material has been demonstrated, this will not impact the suitability for use of the candidate WHO BRM.

Freeze drying allows for the free water to be removed from a solution of a biological substance using vacuum and low temperatures with minimal damage to bioactivity. During the freeze-drying process, water is transformed into ice and then sublimed from ice to vapour phase, then returned to ice on the condenser *(61)*. Freeze-drying processes should be developed by firstly understanding the critical thermal properties of the formulation(s) chosen. Freeze-drying microscopy, differential scanning calorimetry or other suitable methods will allow for the glass transition temperature (Tg′) and any crystallization (eutectic) temperature to be identified *(62, 63)*. These critical temperatures can be used to design the freeze-drying cycle, as the freezing step must be to a sufficiently low temperature to achieve the maximally concentrated state, and be maintained until all of the containers in the batch have equilibrated. Primary drying conditions can then be set, taking into account the critical temperature information and assessing the sublimation rate of the containers, to avoid visual collapse and ensure the production of a stable homogeneous freeze-dried cake. Residual water can be removed by raising the shelf temperature under vacuum to deliver an ambient temperature-stable product that can then be sealed under inert atmospheric conditions to deliver long-term storage stability.

Water rarely if ever actually freezes at 0 °C in a freeze-dried vial and a delay in freezing until a lower temperature is achieved (termed “supercooling”) is always observed. Such delayed nucleation in specific containers is spontaneous and uncontrolled, and a source of heterogeneity in the freezing process. “Induced nucleation” approaches have been described in the scientific literature to address this issue *(64)*. Another approach is to use annealing or thermal tempering *(65)* whereby the use of a plateau raised temperature in the freezing process is introduced to produce greater homogeneity in the nucleation process, and induce the crystallization of excipients that can exist in unpredictable states, such as mannitol or glycine (which may otherwise be found in amorphous or crystalline forms that can affect their capabilities as stabilizing excipients). Induced nucleation or annealing can also result in a reduction in the length of primary drying time required – though the level of moisture remaining after secondary drying may need to be monitored *(66)*.

A.5.3 Distribution into containers

A.5.3.1 General considerations

To ensure homogeneity across the candidate WHO BRM batch, the filled containers 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, unless otherwise justified. To achieve a high precision of fill, the bulk material is dispensed accurately in liquid form into a number of suitable containers. For lyophilized materials, the contents of the containers are then 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 recommended storage conditions.

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

Each container in the batch should either be permanently marked with some form of in-process identification of the material being filled (without impacting container integrity) or a process should be in place to ensure the separation of single runs of different materials.

A liquid bulk representing a suspension or solution likely to display gradient phenomena (for example, due to high sugar content) should be stirred continuously (but gently to avoid excessive frothing which may induce aggregation) during filling and held at constant temperature in order to ensure that homogeneity is maintained throughout the filling process. Exposure to direct sunlight should be avoided. For materials that will be lyophilized, a sample (or samples) of the homogenous bulk material should be removed prior to filling. Such samples can then be used to assess any loss of activity upon lyophilization once the lyophilization process has been completed. Aliquots of these samples can also, on a case-by-case basis, be stored in suitable containers at ultra-low temperatures (for example, in vapour-phase liquid nitrogen) to serve as a benchmark for longer term stability or investigation studies.

Filling should be carried out in a controlled environment – for example, in a clean room or laminar flow cabinet equipped with a high efficiency particulate air (HEPA) filter in order to minimize the risk of contamination.

> Criteria for the quality of the air or for the performance of air filtering systems should be defined, and relevant parameters monitored accordingly.

Since a reference material in the dried state has to be reconstituted, potentially introducing further variability, the precision of fill should be as high as possible to ensure low variation in filling weights across the batch.

> There are no formal pass/fail criteria for the quality control parameters given below. The most important criterion is fitness for purpose in the assay(s) for which the reference material is intended to be used. Nevertheless, the criteria specified below are expectations that are fulfilled by the vast majority of WHO BRM.

A.5.3.2 Liquid fills

For the filling run, an appropriate number of containers should be selected and weighed before and after filling in order to check for variation in the amount (volume or mass) filled into each container. Some filling lines will allow for 100% of the batch to be weighed through automation of the weighing process. However, for other filling lines, a manual process will be needed. Containers should ideally be selected according to a stratified random process to ensure, as far as possible, that the sample is representative of the filling run. The representativeness of these internal process controls (IPC) can be assured by picking the containers at a regular path (batch size divided by the number of IPC). A better estimation of potential process deviation will be achieved by sampling two or three successive containers instead of one at each IPC step. The precision of fill, as measured by the coefficient of variation in filling weights, can be derived from the data obtained. The batch should be assessed for any systematic change in filling weights over the course of the process.

The nature of a liquid influences the precision with which it can be dispensed for filling. Where a reference material is to be freeze-dried, a coefficient of variation not greater than 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 1mL fill of less than 1% is a realistic expectation.

Where a reference material 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, with the suitability of the chosen volume checked by carrying out an extraction test.

A.5.3.3 Powder fills

Powder fills may be used when the amount of material is not a limiting factor, and may be necessary for water-insoluble materials.

In certain cases (such as for some antibiotics), solid bulk materials may need to be filled. In such cases, materials are distributed into glass containers as powders, using manual, semi-automatic or automatic means. Special precautions should be taken to ensure that both the bulk material and the samples taken from it are homogeneous. Homogeneity is achieved through special mixing and sampling devices.

Filling accuracy is maintained through calibrated dispensing tools, with automatic systems validated for precision and repeatability. Manual filling, although subject to variability, is acceptable when an exact quantity is weighed out at the time of use or when the reference material is intended for qualitative use.

A.5.4 Processing of filled containers

A.5.4.1 Freeze drying

The freeze drying of filled containers should be carried out under optimal conditions. It is essential to ensure that all of the containers in a batch are processed together, from the time of

filling until completion of the process, so that they are subjected to the same conditions at the same time. For full-scale lyophilization of most candidate WHO BRM, only one such material should be processed at a time in the freeze dryer as the possibility of cross-contamination cannot be excluded when more than one material is present.

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 immobilized (that is, completely frozen). The temperature for achieving this maximally concentrated stable state (glass transition temperature) is critical to the freeze-drying process and should be determined experimentally. Differential thermal analysis and electrical resistivity are less-sensitive techniques, with differential scanning calorimetry or freeze-drying microscopy more commonly used. Supercooling is a complex phenomenon influenced by many factors and may also differ in degree depending on the container quality, processing conditions and other factors. The liquid in the containers should be frozen to a sufficiently low temperature to ensure that this condition is reached.

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 activity. Some antibodies, clotting factors and enzymes are known to denature under these conditions. Thus, the rate and temperature at which freezing is carried out are important in preserving the activity and solubility of the material, and the most suitable conditions should be determined experimentally. The formulation of the product may help to circumvent these issues. In some cases, the precise conditions required for successful freeze drying of a given liquid can only be deduced from experience with similar materials.

The duration of the freeze-drying process should be validated and extend well beyond that found experimentally to be the minimum necessary, as the temperature gradient between the walls of the chamber and the centre of a shelf can result in different rates of freeze drying. It is well demonstrated that the last containers to dry are those in the centre of the trays, and so drying should be sufficient to ensure that all containers have reached the end of the process. Drying performance will also differ between dryers, and the capabilities of the dryer should also be considered when moving from trial to production processes. There are a number of process analytical technologies which can be used to assure freeze-drying processes *(67)*. Freeze drying is essentially a three-step process: (a) freezing to ensure total immobilization of the solution to be dried, including annealing steps if crystallizable formulants such as mannitol are present; (b) primary drying where ~90% of the water is removed, usually at sub-ambient temperatures; and (c) secondary drying where the remaining water is removed in order to deliver a product which is storage stable at ambient conditions.

The filled containers are usually processed in a shelf freeze dryer. The containers are arranged on trays from which the base can be withdrawn (which aids thermal conductivity during the

process), on temperature-controlled shelves or by automatic loading in an evacuated chamber. In some cases, the temperature of the material in the containers is recorded continuously. When 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. The sublimation of water draws heat from the material in the containers which is replaced by heat from the shelves.

The technical capabilities of modern freeze dryers (such as low chamber pressure and low condenser temperature) usually remove the need for further drying. Further desiccation was originally introduced because freeze dryers were less efficient and it was necessary to further reduce residual moisture, but this is usually no longer required or practised.

The freeze dryer should be cleaned and sterilized between batches using validated procedures.

All lyophilized materials are hygroscopic. It is therefore essential that containers of the lyophilized candidate WHO BRM 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 the uptake of moisture and oxygen *(68)*.

The containers should be sealed in such a way as to preserve the integrity of the contents over the intended lifetime of the WHO BRM. Ampoules should be sealed by fusion of the glass by drawing either under vacuum or after filling with dry nitrogen. Non-invasive measurement of headspace gas as a means of determining integrity can be performed using frequency modulated spectroscopy or other methods to determine residual oxygen.

If not done at the filling stage, the sealed containers should be permanently marked with some form of in-process identification (without impacting container integrity) or a process should again be in place to ensure the separation of single runs of different materials. The sealed containers should be stored at an appropriate temperature and protected from light.

A.5.4.2 Procedure where freeze drying is not used

Containers filled with the appropriate amount of liquid or powder should be sealed (ampoules) or stoppered and crimped (vials) to ensure closure integrity. The same sealing precautions described above for freeze-dried materials should be followed to ensure product stability over its intended storage conditions and lifetime. Stoppering, crimping and sealing unit operations are performed on an automated machine to guarantee reproducible closure throughout the batch. The machine is ideally the same as the one used for filling unit operation and is equipped with precision needles capable of dispensing inert gas before, during, and after the filling process if necessary. This ensures protection against oxidation and moisture contamination. For oxygen-sensitive and hygroscopic materials that require filling in a glovebox, stoppering is carried out in the same environment.

A.6 Quality control of candidate WHO BRM

Testing for microbial contamination should be performed using representative containers from the batch. Samples of the pre-filled bulk material should also be tested to help identify potential sources of contamination where evidence is found. The residual moisture content and headspace oxygen content of the lyophilized final candidate WHO BRM in the container should also be determined. While there are no formal pass/fail criteria for these two parameters (because the ultimate criteria to be met are stability of the material and its fitness for purpose), the levels for residual moisture content referred to below are met by the vast majority of WHO BRM. The following considerations are also applicable to final candidate WHO BRM in powder form.

A.6.1 Residual moisture content of lyophilized materials

Excess free water may adversely affect the stability of lyophilized biological materials *(69–72)*. For this reason, measuring residual moisture content is an important quality control parameter for lyophilized candidate WHO BRM. This is determined using final containers in order 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 will depend on the test methods to be used and on the size of the batch, and should be determined based on a predefined sampling plan. Various methods of moisture determination are available, of which the coulometric Karl Fischer method is most widely used. The impact of residual moisture on stability will be material specific. Materials with a moisture content of less than 1% (w/w) have typically shown adequate long-term stability. Higher values may be acceptable in some cases if assurance regarding the stability of the candidate WHO BRM has been obtained from stability studies (see section A.7 below).

> An example was the Fifth WHO International Standard for follicle-stimulating hormone and luteinizing hormone (human, urinary) for bioassay, which had a residual moisture content of 2.84%. However, accelerated degradation stability studies indicated no loss of activity at temperatures of up to 37 °C for 6 months, with a predicted loss of only 0.001% per annum when stored under the recommended storage conditions of −20 °C *(73)*.

Since lyophilized materials are hygroscopic, precautions are necessary to avoid moisture uptake during the measurement procedure. When low fill masses are delivered (for example, 10 mg or less per container) the measurement of water content may be challenging due to the limit of sensitivity of the analytical method. In some cases, non-invasive methods of moisture determination may be suitable, and these have been used where the infectivity of materials precluded destructive methods. Water activity determination based on the non-destructive analysis of headspace moisture is becoming popular *(74, 75)* and may be more relevant to the stability of biological products than total water content determination (where salt hydrates, which may not impact the stability of amorphous materials, are also included in the measurement).

A.6.2 Residual oxygen content

Residual oxygen may adversely affect the stability and activity of lyophilized materials. The atmosphere within the container needs to be inert (for example, argon or nitrogen) and the material protected against oxidative damage.

The number of containers to be tested will depend on the test methods to be used and the size of the batch, and should be determined based on a predefined sampling plan. Non-invasive oxygen determination using frequency modulated spectroscopy has been applied to measure residual oxygen content *(76)*. This method is more practical and non-destructive, meaning that the same containers can subsequently be used for other purposes (such as bioactivity testing or residual moisture determination) after the non-invasive oxygen measurements have been made. Although studies have shown that residual oxygen may not be deleterious for some reference materials *(77)*, it should be borne in mind that where a higher oxygen content is indicative of atmospheric air ingress (of typical relative humidity), the moisture content will also rise.

A.6.3 Characteristics and activity

It is essential that the biological material in the container is demonstrated to have retained its integrity, composition and activity, using appropriate methods. For freeze-dried materials, comparison against the (appropriately stored) liquid bulk can provide valuable information on any loss of activity as a result of the production process.

A.6.4 Considerations for homogeneity assessment

The vast majority of WHO BRM are lyophilized materials produced as a single batch from a single homogeneous bulk material in one working session. An essential requirement for any WHO BRM is equivalence between the containers, and a well-controlled production process with high precision of the filling volume is an important factor in ensuring the homogeneity of the finished candidate WHO BRM (see A.5.3.2 above).

Where possible, an assessment of homogeneity should be conducted to demonstrate that the candidate WHO BRM is sufficiently homogeneous with respect to the measurand for which it is intended to be established. However, in situations where the analytical methods used are imprecise (for example, an in vivo bioassay for vaccine potency), it may not be possible to perform such a study with sufficient statistical power to distinguish any apparent batch non-homogeneity from poor method repeatability. In these cases, measurement of another property of the candidate WHO BRM (using a more precise analytical method) may be useful for the purpose of demonstrating homogeneity.

In cases where it is possible to obtain suitable estimates of activity for a sufficient number of containers tested under strict repeatability conditions (that is, the same precise measurement procedure, same operator, same equipment under the same conditions, same location and repetition over a short period of time) then the methods recommended in ISO 33405 *(78)* may be used to assess the inter-container variation. Containers should be selected using a random

sampling process covering the whole production batch. The number of containers selected will depend on the batch size, and at least the cube root of the total batch size is recommended for testing (that is, a minimum of 10 containers for batch sizes below 1000 containers). The data obtained from the homogeneity assessment are an important part of the overall package of evidence required to demonstrate that a candidate WHO BRM will be fit for purpose.

A.7 Stability assessment

A.7.1 General considerations

Determination of the stability of a candidate WHO BRM under a variety of conditions is recommended to:

- provide assurance, including at the time of proposed establishment, that the material is likely to retain its activity and remain suitable for its intended purpose under its defined storage conditions;
- define appropriate conditions for the distribution of the WHO BRM to users following its establishment; and
- determine the extent to which the WHO BRM will remain suitable for use over time following reconstitution.

The selection of suitable analytical methods for monitoring or predicting stability will depend on the nature and intended use of the WHO BRM. Approaches to stability testing typically focus on the activity of the WHO BRM in the analytical procedures that it is intended to be used for. However, additional stability-indicating methods may also be performed to support stability conclusions. In the case of assays with lower precision, it might be useful to apply an isochronous study design, as described in ISO 33405 *(78)*. This allows different storage conditions and times to be assessed in one measurement series (under repeatability conditions), which is expected to lead to improved measurement precision and improved ability to detect any changes in activity.

It is important to recognize that in most cases, WHO BRM are of the highest metrological order and serve as primary reference materials. This means that they define the IU for the activity being measured and, critically, that there is no higher order reference material against which their stability can be measured. Equally, for biological analytes, it is not generally possible to describe a reference method that can be performed independently of the reference material in such a way that it can be used as a baseline measure of stability. It is therefore the case that no direct method of monitoring activity, or estimating the rate of loss of activity of the reference material under its defined storage conditions, is possible and approaches must be based on a protocol that tests the material against itself (under different storage conditions) or against suitable comparator preparations (to monitor their relative activity over time). While providing useful guidance, stability testing paradigms developed in related areas, such as those for pharmaceutical substances under the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH), cannot be directly applied to candidate WHO BRM as such approaches typically depend on the existence of a higher order

reference material against which the substance being tested can be compared, a defined traceability chain to the SI unit, or the ability to define the quantity being evaluated by an RMP.

> Previous experience with similar reference materials and formulations showing acceptable stability can be used as supporting information for the likely stability of newly proposed WHO BRM.

A.7.2 Real-time stability monitoring

Strategies for evaluating real-time stability typically focus on monitoring the activity of the reference material relative to one or more comparator preparations. Any significant changes in relative activity are taken as an indication of potential instability in one (or more) of the materials tested. Examples of such comparators include samples of the same candidate WHO BRM stored at ultra-low temperatures (for example, −70 °C or −150 °C), suitable independent preparations (for example, commercial biological products with expected high consistency of specific activity), a previous reference material or other independent materials from previous studies. When selecting suitable materials, the similarity of dose–response curve characteristics is an essential consideration.

> An example of an independent comparator is a normal plasma pool used in the stability testing of candidate WHO BRM for blood coagulation factors.

The frequency of testing and acceptance criteria applied to the relative activity estimates should be determined on a case-by-case basis and should take into account the precision of the assay used, with assays of higher precision preferred. The use of assays of lower precision would require replicate testing to detect small changes in activity.

> It should be recognized that no change in relative activity will be detected if two different materials have the same long-term stability profile and undergo the same rate of change in activity over time. The resulting risk of not detecting a loss of activity in the candidate WHO BRM can be reduced if multiple comparator materials are used and their relative potencies monitored over time.

> In situations where no suitable comparator materials are available, real-time monitoring of assay response variables (such as effective dose 50 (ED50) or cycle threshold (Ct) values) may provide useful evidence to support the stability of the candidate WHO BRM. Trending of dose–response curve parameters can also be useful in this regard – though it is recognized that considerable inter-assay variation in these variables may be present and that instability may not be detected in such cases.

A.7.3 Accelerated degradation stability studies

The basis of stability prediction is an accelerated degradation study in which long-term stability is inferred from data obtained over a much shorter testing period. Samples of the candidate WHO BRM to be tested are stored at elevated temperatures (typically at 4 °C, 20 °C, 37 °C, 45 °C and 56 °C). The activities of these samples are then determined against those of samples stored constantly at −20 °C or lower, typically after 6 months to 2 years.

WHO BRM are typically very stable and exhibit only small losses of activity, even at elevated temperatures. The storage time chosen for the stability study is therefore a compromise between the need to induce measurable degradation and the need to complete the project in a timely manner. It should be noted however that significant loss of activity will often not be observed at 6 months of storage. As a result, leaving samples at the elevated temperatures to be tested at later time points (for example, after 2 years) – which may be after formal establishment of the WHO BRM – is a useful practice.

Indirect and approximate methods can be used to predict the rate of loss of activity in some cases. These methods are generally based on the relationship between reaction rates and temperature given by the Arrhenius equation, with a first order reaction rate frequently assumed. Use of these methods requires that samples of the candidate WHO BRM are stored at a range of elevated temperatures and tested for activity relative to the same material stored at lower temperatures.

> An iterative procedure for predicting the stability of a reference material based on a maximum likelihood approach to estimate the parameters of the Arrhenius equation relating degradation rate to temperature has been described *(79, 80)*. This approach makes certain assumptions which may not always be true for lyophilized materials – in particular, that the degradation process is a first order reaction. Moreover, there are some classes of material for which this equation is clearly not applicable – for example, some preparations are stored as liquids and behaviour may differ markedly between the liquid and frozen states. Live viral vaccines are another special case.
>
> Biological materials may exhibit Arrhenius-type behaviour over a limited 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 materials are designed to be stable under defined storage conditions and may also show no apparent loss of activity after storage at elevated temperatures. However, experience has shown that for some reference materials, reconstitution may be difficult following high-temperature incubation. These and other 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 IS.

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 WHO BRM. Such data may be used to define appropriate distribution conditions.

A.7.4 Further considerations

WHO BRM are expected to remain suitable for their intended use over their lifetime, which may be 20 years or more in some cases. Expiry dates are not assigned to WHO BRM because assurance regarding their long-term stability is a key factor in the ECBS decision to recommend their establishment in the first instance. The ECBS may request further stability prediction studies and/or periodic monitoring of real time stability after establishment, on a case-by-case

basis and depending on the nature of the material and available stability data at the time of establishment.

Following establishment, feedback from the end users of WHO BRM is encouraged and any concerns raised regarding the stability of the WHO BRM should be investigated by the custodian laboratory to determine whether corrective actions are needed. Corrective actions by the custodian laboratory may include communication of updated information related to stability to end users, changes to transport conditions and/or changes to the recommended storage temperature.

Where evidence is available, information on the stability of the WHO BRM after reconstitution should also be provided to end users. However, stability-after-reconstitution studies will not always be conducted by the custodian laboratory and may not always be possible prior to establishment of the WHO BRM. Information on other factors that may affect the properties of the reconstituted material (for example, adsorption onto particular container types) should also be provided where available.

A.8 International collaborative study

An international collaborative study of a candidate WHO BRM is a scientific study designed to provide evidence of its suitability for its intended use. Collaborative studies provide valuable scientific information on the candidate WHO BRM and on the assay systems in current use that could not be obtained by any one individual laboratory. An international collaborative study must be carried out before any candidate WHO BRM can be recommended by the ECBS for establishment. The amount of work and resources required to carry out such a study should not be underestimated.

WHO may, either through the ECBS or through the activities of working groups with relevant expertise in specific areas, make recommendations on the broad outline of the collaborative study to be undertaken.

Collaborative studies should be organized by one or more scientists familiar with the appropriate biological field, working closely with an experienced statistician, and should be conducted according to the principles set out below.

A.8.1 Aims of a collaborative study

The purpose of a collaborative study is to demonstrate that the candidate WHO BRM is suitable for its intended use. Potential aims of the study include the following, though not all of these will be applicable to all standardization projects:

- confirmation that the candidate WHO BRM has the expected properties and activity (or activities);

- demonstration that the candidate WHO BRM is suitable for the calibration of other reference materials, and/or for evaluating products from a variety of manufacturers or other sources;
- assessment of commutability with representative clinical samples (for candidate WHO BRM intended for diagnostic application);
- comparison of two or more candidate WHO BRM;
- assignment of an activity/activities or other parameters to the candidate WHO BRM;
- assessment of performance and suitability for use in different assay methods, including whether different assay methods (such as bioassays, immunoassays, biochemical assays, and in vivo and in vitro assays) measure the same or different properties of the candidate WHO BRM;
- assessment of the molecular integrity and composition of the candidate WHO BRM; and
- assessment of the stability of the candidate WHO BRM.

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

A.8.2 Planning and design

Although there is no generic collaborative study design, the principles set out below should be followed.

The details of the proposed collaborative study, including its underlying scientific rationale, should in all cases be recorded, and these records retained throughout the lifetime of any subsequently established WHO BRM.

> Each study is unique and requires current scientific knowledge on 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 and assay methods. This requires both a biological scientist and a statistician, ideally with experience of such studies, to bring together knowledge of the biological material and the assays used for it. The rationale for the proposed study design and the proposed statistical methods to be used for analysing the study results should be outlined.

A key decision that will influence the study design is the choice of unit (IU or SI) to be assigned to the candidate WHO BRM. The choice of unit and supporting rationale should be explicitly stated in the study protocol.

It is also necessary to determine which samples will be included in the study in addition to the candidate WHO BRM (see section A.8.4 below). The study should be designed so that each assay generates data allowing for assessment of statistical validity (for example, evidence of similarity of dose–response relationships) and precision *(81)*.

> When the capacity of the study allows, the inclusion of coded (blinded) duplicate samples can be a useful aid in assessing the acceptability of data returned by individual study participants.

The data from testing such samples may also be useful in determining similarity equivalence ranges as part of the study analysis.

For candidate WHO BRM intended to have diagnostic application, an assessment of commutability should be included in the study. Guidance on the design of the commutability study and its evaluation is provided in "General considerations" above and in section A.8.8 below. Commutability studies typically require the inclusion of a suitable number of representative clinical samples. However, even where the availability of representative clinical samples is limited, an indication of commutability can still be obtained by including at least some such samples in the study.

The number of study participants will depend on the nature of the study, taking into account its aims, the type of candidate WHO BRM being evaluated, the number of different assays or platforms to be covered, and the required representation of different geographical regions and user groups (for example, control laboratories, health care laboratories, academia and manufacturers) (see section A.8.3 below). The majority of studies are likely to involve between five and 25 participants.

For the evaluation of a proposed new WHO BRM, it may be necessary to include a wider range of assay methods in the study compared to a study for a proposed replacement WHO BRM using an established assay procedure or small number of such procedures.

For example, in collaborative studies of proposed replacement WHO BRM intended to be used for IVD calibration, well-known assays with quantitative output and sufficient linear range may be preferred to qualitative assays testing limiting dilution series, which are often associated with greater variability of the results.

As a result, collaborative studies for proposed new WHO BRM may require more participants compared to studies for a replacement WHO BRM. However, as outlined above, the overall size of any given study will be influenced by several factors. In the case of replacement IS that define the IU, ensuring continuity of the unit will be a key consideration when determining the number of participants and method types to include in the study. In some cases, this may require prequalification of participating laboratories to demonstrate proficiency in the relevant analytical method(s). Furthermore, consistent analysis of the raw data received from all study participants should be performed centrally by the coordinating laboratory, and suitable statistical methods for deriving a robust assigned value should be applied.

If a new WHO BRM is to be established with a defined unit of activity, a method for measuring the desired activity should already exist and be used. If several assay methods are available, the candidate WHO BRM chosen should be suitable for use with as many of these as possible. For some studies, participants may be asked to perform more than one method, and in some cases to perform an additional method based on a protocol shared by the custodian laboratory. If it is intended that participants use the same assay method, a protocol for the procedure and

any critical reagents (as well as a sufficient number of study samples) should be provided and sufficient time allowed for laboratories to become familiar with the method.

> Where appropriate, working groups may be formed to facilitate the development of standard procedures. Guidance may be provided by the working group on the method to be used and selection of laboratories.

Study participants will be asked to carry out a specified minimum number of independent test runs which, based on knowledge of the assay reproducibility, could be chosen to ensure mean estimates of acceptable precision. An independent test run is usually defined as one made using fresh dilutions from a newly opened container or a fresh weighing of each material and carried out on separate days. A duplicate test is a repeat test using the same solutions. Since this does not include all of the variables associated with weighing and dilution, it is not truly independent.

If the study is of a proposed replacement IS, the way in which continuity of the IU will be addressed is a key study design consideration that should be explicitly described in the study protocol. The aim of continuity is to ensure that the IU defined by the replacement IS is consistent with the IU defined by the previous IS. This in turn ensures that measurements made in biological and immunological assays can be compared over time, and merits particular attention if there has been a change in the starting material or formulation between the current IS and the proposed replacement IS.

A.8.3 Collaborative study participants and their role

Study participants are invited based on their experience with the analytical methods that will be included in the study. Participants may come from a variety of sectors, including national control laboratories, product manufacturers, diagnostic assay providers, public health laboratories and academia. The selection of participants is the duty of the laboratory organizing the collaborative study on behalf of WHO. In light of the global mandate and responsibilities of WHO, participating laboratories from as many of the six WHO regions as possible should be involved in the study.

The identification of potential study participants should be carried out using a variety of information sources relevant to the type of laboratories that are expected to use the WHO BRM once it has been established. For proposed new WHO BRM, such sources may include publicly available databases of prequalified therapeutics, vaccines and IVDs (to help identify potential industry participants), and members of the WHO-National Control Laboratory Network for Biologicals and relevant WHO CC networks (to help identify potential participants from national authorities and reference laboratories). Recently published scientific articles may also help in identifying laboratories with the relevant expertise, and therefore potential study participants. For proposed replacement WHO BRM studies, in addition to the above sources, records of the users of the current WHO BRM and of participants in the earlier study conducted to establish it, are also valuable sources of information that can help identify potential

participants. WHO, through its regional offices and members of expert advisory panels, may also be able to help identify potential study participants.

When invited to take part in the study, potential participants should be provided with an overview of the study and its aims, together with details of the number of samples to be tested and the number of assays to be performed, as well as the estimated timelines for the study. This will be important in enabling invited laboratories to make an assessment of the resources required to participate. Potential participants should be asked to indicate:

- the assay methods which they could undertake;
- whether they have the capacity to perform the number of independent runs requested for the proposed number of samples in each of the methods to be used;
- whether the laboratory operates under an accredited or other quality management system:

  The presence of a quality management system does not guarantee the quality of the data submitted – only an appropriate study design and proper assessment of the collaborative study data can assure data reliability.

  The methods used by participating laboratories should be qualified and appropriate for the intended use. Prior to participation in a collaborative study, participants may be requested to undertake proficiency studies, and this may be particularly relevant for studies leading to the assignment of SI units to a proposed WHO BRM. As a rule, where SI units are assigned to a proposed WHO BRM, they should be derived from validated physicochemical procedures with acceptable performance characteristics.

Participants should also agree:

- to complete their studies within the timelines specified;
- to report their raw data using the reporting form supplied;
- to accept responsibility for the safe handling and disposal of the study 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 provider of a collaborative study sample and WHO, as a condition of participation.

- not to publish information on a proposed WHO BRM without the prior agreement of WHO, since the premature publication of such information prior to establishment can cause scientific confusion:

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

Participants will be asked to provide comments on the draft collaborative study report before its submission to WHO. Participants will be listed in an appendix to the report (for example, alphabetically by country) but will be denoted only by a code number or letter in the main section of the report (and only where this is necessary) in order to retain anonymity.

A.8.4 Materials to be included in the collaborative study

Materials provided by the coordinating laboratory for use in the collaborative study may, in addition to the candidate WHO BRM, include reference materials in current use, coded duplicate samples, and frozen liquid counterparts of lyophilized materials. In some cases, typical samples (such as clinical samples) for which the proposed WHO BRM is intended to be used (or one or more dilutions of such a sample) will also be provided to assess commutability. In some studies, participants will be asked to test materials that are not provided to all participants by the coordinating laboratory. Examples include in-house standards or, in the case of manufacturers, representative batches of their own product. Such materials are particularly useful in studies for some replacement WHO BRM and can provide additional information on the impact of replacing the current WHO BRM.

> Normal plasma pools may be included in studies of candidate WHO BRM for blood coagulation factors to provide assurance regarding continuity of the IU. Where this is done, the study report should provide details of the donor pools used to obtain the normal plasma pool.

To avoid potential bias, all study materials should be blinded (for participants) and labelled with an appropriate code so that participants cannot identify materials or their sources.

A.8.5 Distribution of materials

The study materials should be distributed to participants in accordance with current postal or air freight regulations *(82)* (see section A.10.3 below). 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. Materials should be accompanied by directions for their storage, handling and safe use, and disposal. Participants should be requested to report the condition of the materials immediately upon receipt to the study organizer.

> Temperature monitoring devices may be included with the shipment, or on the label of the materials. If any concerns regarding the condition of the materials are reported, the study organizer should make a decision as soon as possible as to whether or not there is a need to ship replacement materials, and should inform the participant accordingly.

A.8.6 Reporting of results

Each participant should be provided with clear guidance on what should be reported to the coordinating laboratory. This can include information on:

- the assay method(s) used, including details of the assay design and layout – this may also include details of any animals used (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 used for making dilutions of test samples and reference materials – this is important for the calculation of results and the identification of possible causes of variation, bias or inaccuracy; and
- assay results given as raw (that is, unprocessed) data – all data obtained should be reported and an explanation given for the proposed rejection of any data.

A template spreadsheet provided to study participants with a consistent format for reporting results can be useful to aid the study analysis and ensure that all relevant information is reported. In addition, participants should be encouraged to provide their own statistical calculations as this will help to show whether they interpret their results in the same way as the statistician responsible for the overall study analysis.

A.8.7 Analysis of results

Results from all participants are analysed by statistical methods described and considered appropriate by the statistician responsible for the study, who should be experienced in the statistical evaluation of the results of various assay types. The data from each laboratory should be analysed separately, and data validity and acceptability evaluated. Any issues or questions arising should be promptly discussed with the participant laboratory concerned.

The variability in the results obtained both within and between laboratories, and between assay methods, should be assessed as part of the analysis, and the outcomes of such an assessment used to support an appropriate combination of estimates from all valid assays. There are no generic outlier detection rules, and the rationale for the identification and exclusion of any results should be described and justified.

> Assay results should also be displayed graphically (for example, as histograms) as this may help to detect unusual features that may be overlooked in the study of numerical data alone.

A.8.8 Specific considerations for the assessment of commutability

Although the specific considerations described below relate to the assessment of commutability for a candidate WHO BRM with a diagnostic application, some of the principles may also be relevant for other types of WHO BRM.

A.8.8.1 Commutability assessment of proposed new WHO BRM

Assessing commutability by testing representative clinical samples in multiple assays (see also "Commutability" in General considerations above) is a resource-intensive enterprise that could be undertaken either as part of the collaborative study or in a separate study. Where multiple assays can be performed by a single laboratory (for example, using commercially available measurement procedures), it may be possible for the assessment of commutability to be conducted by that laboratory, provided a suitable number of representative clinical samples can be obtained.

Relevant factors when planning a commutability study include:

- relatedness of the candidate WHO BRM matrix to the usual matrix of clinical samples;
- potential interference of preparation steps and materials (for example, lyophilization, stabilizers and preservatives) with the measurand and/or assays;
- potential diversity of the measurand (for example, of viral genotypes in virus detection assays);
- number of different methodologies and number of different assays available for the measurand;
- developmental stage of assays; and
- global market share of individual assays.

In biological measurements of clinical samples there is often no RMP available. In this situation, commutability is often assessed by comparing the results obtained by multiple different assays designed for the same measurand. This is done by parallel testing of a panel of representative clinical samples (reflecting different physiological concentration levels of the measurand) and the candidate WHO BRM at different concentration levels of the measurand. Measurand concentrations at levels relevant for medical decisions should be included in the commutability study.

> Several approaches to the statistical assessment of commutability studies exist. One approach is to select a set of native clinical samples spanning the relevant concentration interval and analysing both the candidate WHO BRM and the clinical samples using multiple assays. Random error is minimized by performing measurements in single runs and in replicates. Data for each assay pair are then analysed by Deming regression analysis to determine if the candidate WHO BRM behaves like the clinical samples (for example, by falling within the 95% prediction interval defined by the clinical samples) *(83)*. Another approach is to quantify the systematic difference between the results of two assays (bias) for the clinical samples and comparing this with the bias obtained for different concentrations of the candidate WHO BRM. The reference material is considered commutable if the difference in bias is smaller than a predefined commutability criterion *(84)*. An approach usually applied to data from WHO collaborative studies that include a large panel of clinical samples considers the calibration effectiveness of the candidate WHO BRM in the harmonization of assays. This is done by demonstrating that the inter-assay variation for clinical sample results obtained when using the proposed reference material as a calibrator is within an acceptance criterion ideally defined for each measurand based on the requirements of the clinical application *(85)*.

A.8.8.2 Commutability assessment of proposed replacement WHO BRM

Typically, the characteristics of a proposed replacement WHO BRM (including the source and composition of the analyte, the matrix composition and the preparation steps) will be similar to the current WHO BRM. In such cases, commutability information gained during the establishment of the WHO BRM may in principle be assumed to also be applicable to its proposed replacement, and a less extensive commutability evaluation may be justified, preferably with a focus on assays not previously included *(86)*. However, in cases where the analyte composition, matrix and/or essential preparation steps are different for the proposed

replacement WHO BRM, and/or critical feedback concerning commutability has been obtained for the current WHO BRM, more extensive efforts should be undertaken to assess the commutability of the proposed replacement.

A.8.8.3 Commutability conclusions

Conclusions regarding the likely commutability of a candidate WHO BRM should be based on an overall assessment of data for the laboratories and assay methods included in the collaborative study. The commutability criteria used and how they were derived should be clearly described in the study summary report (see section A.9 below). While limited commutability data for some assays will be available from the collaborative study report, users should be encouraged to assess the commutability of the new or replacement WHO BRM, once established, in their own assays. Such information is essential since the potential non-commutability of any given reference material for a particular assay might be managed in some cases by using an assay-specific correction factor *(29)*.

The inclusion of candidate or already established WHO BRM, along with representative clinical samples, in external quality assessment schemes has been shown to be another useful data source for gathering information on commutability and for identifying any potential issues related to non-commutability.

A.9 Summary report for submission to WHO

A comprehensive summary report describing the preparation and characterization of any proposed WHO BRM must be submitted to WHO. The report should include a table of characteristics for all candidate WHO BRM that includes target and obtained values for precision of fill (expressed as the coefficient of variation (CV%) of the fill mass), and additionally for lyophilized candidate BRM, target and obtained values for residual moisture and oxygen headspace (see Table A1.1 in Appendix 1). Where target values were not met, considerations regarding the fitness for purpose of the proposed WHO BRM should be included in the report.

An explanation of the geographical distribution and degree of regional representativeness of study participants should also be included, particularly in cases where such representativeness was limited. More detailed guidance on the information to be included in the initial summary report (which will become the subsequent BS summary report following the assignment of a WHO BS number – see below) is provided in Appendix 1 of this document.

For proposed WHO BRM that have an assigned value, the initial summary report should include a clear proposal for the value(s) to be assigned together with the rationale, including whether the proposed value is restricted to certain methodologies (for example, neutralization assays for an antibody WHO BRM). The naming of proposed WHO BRM should comply with the considerations and conventions outlined below in Appendix 2.

The proposed value(s) to be assigned to the WHO BRM at establishment must be expressed in terms of the container (typically vial or ampoule) for lyophilized materials or per mL for liquids. For solid reference materials, the proposed value should be expressed as the mass containing 1 IU, or as the number of IU/mg (as is the case, for example, for some antibiotic IS).

Prior to submission to WHO, the summary report should be sent to each collaborative study participant, together with the code used to identify them. The participants should then confirm or comment upon whether:

- their data have been correctly interpreted in the analysis;
- they agree with the conclusions drawn and recommendations made in the report, and that the proposed WHO BRM will be suitable to serve its defined intended purpose; and
- the proposed unitage is appropriate.

Any disagreement(s) expressed by collaborative study participants should be noted, together with any relevant critical comments, for consideration by the ECBS.

The resulting summary report, amended where necessary and indicating the agreement or otherwise of collaborative study participants with its content, should then be submitted to WHO.

The submitted summary report is posted by WHO on its website for public comments and assigned a BS document number. The BS summary report then serves as the basis of the respective presentation to the ECBS. The BS summary report can, if necessary, be updated in light of any amendments proposed by the ECBS, and is in all cases referenced in the corresponding entry in the WHO BRM catalogue.[2] Through this link, the respective BS summary report can be downloaded from the WHO website. Authors are strongly encouraged to submit a revised version of the BS summary report for publication in a peer-reviewed scientific journal. A manuscript submitted for publication should report the outcome of the ECBS recommendation, and present the associated data and methods used in a more concise manner.

Once the proposed WHO BRM has been established, the BS summary report (further updated, if necessary, in light of any amendments requested by the ECBS) should be used as the basis of the IFU that accompanies every shipment of the WHO BRM. Further guidance on the information to be included in the IFU is provided below in Appendix 3.

The data used to support the establishment of a WHO BRM can be made available to users either through reference to the respective scientific publication describing its establishment or

[2] Available at: https://www.who.int/teams/health-product-policy-and-standards/standards-and-specifications/norms-and-standards/catalogue.

through the final version of the BS summary report, or both. A reference to the final BS summary report should be included in the accompanying IFU (see Appendix 3).

A.10 Labelling, storage and distribution of WHO BRM

A.10.1 Labelling of WHO BRM

Following establishment, each container in a WHO BRM batch should be labelled to show the following information:

- the name "WHO";
- the name of the WHO BRM in the form "First [or "Second" etc.] WHO International Standard [or WHO International Reference Panel or WHO Repository] for …" or [no ordinal number] "WHO International Reference Reagent for …";
- the unique code allocated by the filling laboratory to enable the batch to be identified;
- the recommended storage conditions; and
- a statement that the material is not to be administered to humans.

If the size of the label permits, the following information may also be shown:

- the year in which establishment of the WHO BRM was recommended by the ECBS;
- the value(s) assigned to the WHO BRM; and
- the name and address of the custodian laboratory designated to store and distribute the WHO BRM.

If the size of the label is not sufficient, this information must be shown in the IFU that accompanies the WHO BRM.

A.10.2 Storage of WHO BRM

Custodian laboratories store WHO BRM on behalf of WHO. The custodian laboratory for each WHO BRM is identified in the online WHO BRM catalogue.[3] A key responsibility of the custodian laboratories is to maintain the integrity of the stored materials. This includes the monitoring of storage temperature with alarm systems, and having protocols and procedures in place to respond to any alerts indicating deviation from the predefined temperature limits. Systems should also be in place to prevent accidental or intentional tampering with freezer or alarm settings. Custodian laboratories should provide training for relevant personnel in maintaining the low-temperature storage of reference materials, and should have comprehensive contingency plans to ensure that storage integrity is maintained, including the use of back-up generators that will provide power to cold storage units in the event of a mains power failure *(87)*. Custodian laboratories are also encouraged to implement and maintain the

[3] Available at: https://www.who.int/teams/health-product-policy-and-standards/standards-and-specifications/norms-and-standards/catalogue.

off-site storage of sufficient numbers of each IS to allow for establishment of a replacement IS in the event of catastrophic loss of, or damage to, the entire storage facility.

A.10.3 Distribution of WHO BRM

Custodian laboratories should ensure that appropriate precautions are taken to ensure that shipments of WHO BRM comply, where applicable, with international regulations on the transport of infectious substances *(82)*. When shipping biological materials from one country to another there are several import/export considerations that may apply, including:

- Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) – although uncommon, controls for the protection of endangered species may apply to animal-derived components if the donor animal is CITES listed.

  For example, VERO cells are subject to CITES permits due to them originally being obtained from *Chlorocebus aethiops*. Similarly, some older reference materials are subject to CITES due to them containing serum from protected species.

- Animal by-products – products which contain or are derived from animal materials may be subject to zoo sanitary controls due to being a potential vector for animal pathogens. This will be especially the case where the donor animal is a "livestock species" such as poultry and ungulates.

  Examples of such components are fetal bovine serum, bovine serum albumin, porcine mucosal heparin and material grown in hens' eggs. The scope and level of control will vary between countries and so importers will need to consult with their local national authorities.

- Import permits – where controls apply, importers are likely to require import permits, often supported by formal declarations provided by the shipper attesting to the health of the donor animal and/or inactivation and sterilization methods used.
- Infectious agents – some countries may place additional import controls on materials that contain human pathogens. This is less common than zoo sanitary and animal pathogen controls but importers should check with their national authorities. It should also be noted that infectious agents when shipped must comply with "Dangerous Goods" regulations which require special training, packing and transport handling.
- Genetically modified organisms (GMO) – import and export permits may be applicable to such organisms or to genetically modified elements such as recombinant DNA, RNA, plasmids or vectors that contain or express the modified sequences.
- Strategic export controls – some countries will have export and import controls applicable to materials which are deemed to be of potential biosecurity concern. This includes a number of import entries for viruses, bacteria and derived toxins. Notably such controls may also apply to DNA, RNA or other genetic elements derived from, or encoding for, a listed import entry. Where a material is in scope, licences would need to be obtained by the exporting and importing parties.

Relevant safety information should be provided to recipients following the globally harmonized system (GHS) for hazard classification and labelling. A safety data sheet is a

standardized detailed informational document for a potentially hazardous material that describes the relevant properties of the material, including its potential infectivity and pathogen risk group classification. The safety data sheet is prepared by the custodian laboratory and provided to WHO BRM recipients.

WHO BRM are not usually intended for routine use in every test run and, in some cases, custodian laboratories may limit the number of containers of a WHO BRM that can be provided to any given user within a defined period. The custodian laboratory should consider the intended use of the reference material, its batch size and likely rate of use in situations in which restrictions may need to be put in place. WHO BRM should be managed in such a way as to ensure that the need for their frequent replacement is avoided (see section B.2 below).

WHO BRM may be distributed free of charge to national control laboratories and/or international or regional standardization bodies for their intended purpose.

Rules to prevent the commercialization of WHO BRM should be clearly set out in the IFU (see Appendix 3 below) and/or in the terms and conditions under which the WHO BRM is provided by the custodian laboratory.

Part B. Use of WHO biological reference materials

B.1 General considerations

The intended use of any given WHO BRM should be defined in the respective IFU (see Appendix 3 below) provided with each shipment. The IFU should also be available online through the custodian laboratory website. Most IS are intended for the calibration of secondary standards as outlined below in Part B.2 and in the corresponding WHO manuals for secondary standards *(12–14)*.

WHO BRM can also support assay development, validation and monitoring, with some IRR and IRP established specifically for these purposes. Although IS may also be useful for these purposes and for other purposes (such as monitoring the stability of secondary standards), custodian laboratories may not be able to guarantee the supply of an IS in the quantities needed for such uses. The stocks of an IS need to be carefully curated to avoid the need for its frequent replacement. This will minimize the number of complex and resource-intensive collaborative studies required, as well as minimize the potential regulatory burden for users resulting from IS replacement. Where large quantities of an IS are requested, the custodian laboratory may ask for supporting justification for that request, and may need to limit the number of containers provided depending on the intended use and available stock of the IS.

When a replacement WHO BRM is established by WHO it will be made available by the custodian laboratory and added to the online WHO catalogue.[4] In some cases, the replacement WHO BRM may not be made available immediately if sufficient stocks of the current WHO BRM are still available. In such cases, the date of inclusion of the replacement WHO BRM in the WHO catalogue will be agreed with the respective custodian laboratory. As soon as a replacement WHO BRM is made available by the custodian laboratory, the previous WHO BRM should be removed from the custodian laboratory catalogue. When a replacement IS becomes available, the need for, and timing of, recalibration of existing secondary standards and/or assays should be determined by users on a case-by-case basis, taking into consideration the purposes of the testing performed.

Laboratories that retain a local stock of the previous IS may decide to continue to use that IS (for example, to continue monitoring the stability of a local reference material for which limits have been established based on the previous IS) subject to local regulatory and Quality Management System (QMS) requirements. However, when a new secondary standard needs to be established, or the initial validation data for an IVD need to be determined (or re-determined), this should be done using the replacement IS, unless otherwise scientifically justified and authorized.

When a replacement IS is established, the IU for a given measurand is redefined, and every effort is made through careful collaborative study design and data analysis to ensure the continuity of the IU from the current IS to its replacement. The value assigned to the replacement IS will be based on its behaviour relative to the current IS in relevant state-of-the-art analytical method(s). However, as the continuity of the IU cannot always be robustly assessed for all users, products or assays during the collaborative study to replace an IS, it is imperative that, for IS used in the quality control of biological products, each user independently assesses the continuity of the IU redefined by a replacement IS using their own analytical procedure and product. This will allow for the identification of any potential unintended changes in assay results attributable to the replacement of the IS rather than to changes in the product itself.

Such assessments should ensure that product quality attributes (such as specific activity) remain comparable between commercial batches and batches shown to be safe and efficacious in clinical studies. Where users identify a lack of continuity of the IU following the replacement of an IS used for the quality control testing of a biological product, alternative approaches for the calibration of in-house standards may be considered if scientifically justified by the user and authorized by the national regulatory authority (for example, calibration against a previous, stable in-house standard) to ensure that the quality attributes of the biological product remain comparable to those of batches shown to be safe and efficacious.

[4] Available at: https://www.who.int/teams/health-product-policy-and-standards/standards-and-specifications/norms-and-standards/catalogue.

Any potential observations by users of a lack in continuity of units following the establishment of a replacement WHO BRM should be promptly communicated to the responsible custodian laboratory. Furthermore, users are strongly encouraged to report any other performance issues with WHO BRM (for example, in relation to stability or commutability) to the responsible custodian laboratory, as far as possible supported by relevant data. Advances in analytical technology may also trigger the need to review the suitability, and/or undertake further characterization, of an established WHO BRM.

WHO BRM, or any part thereof, including derivatives (whether modified or not) must not be incorporated into any commercial product.

B.2 Considerations for the preparation, characterization and calibration of secondary standards

B.2.1 Introduction

As supplies of an IS may be limited, regional and national control authorities may consider preparing and establishing secondary standards, calibrated against and traceable to the IS, for wider use. Similarly, a manufacturer undertaking the assay of numerous batches of a biological product will usually calibrate and qualify an in-house standard for routine use in these assays. As with regional and national secondary standards, the unit should again be traceable back to the IS.

The effort involved in developing and establishing secondary standards should not be underestimated. For this reason, countries in a region are advised to collaborate in order to prepare regional secondary reference materials. By doing so, such reference materials are likely to have wider application, with duplication of efforts avoided or minimized.

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

> In Europe, the European Pharmacopoeia biological reference preparations (Ph. Eur. BRP) are "intended for use as stated in a monograph or general chapter of the European Pharmacopoeia". Ph. Eur. BRP are either secondary standards calibrated in IU or preparations which may be used to define a "European Pharmacopoeia Unit" (Ph. Eur. U). Examples include the Ph. Eur. BRP for B19 DNA for NAT testing as a secondary standard *(88)* and the Ph. Eur. BRP for aprotinin solution as a preparation for defining the respective Ph. Eur. U *(89)*. In China, in 2010, in the absence of an IS, national standards for enterovirus 71 vaccine and antibodies were established and assigned a value in units (U), subsequently supporting the development and establishment of WHO BRM for enterovirus 71 in 2015 and 2019 *(90, 91)*.

Many of the principles and considerations set out in Part A of this document also apply to the preparation and characterization of secondary standards. In addition, more specific and detailed practical advice and guidance on the preparation of such standards, traceable to a WHO BRM, are provided in the corresponding WHO manuals on the preparation of secondary standards for

vaccines *(12)*, for IVDs based on nucleic acid or antigen detection *(13)* and for antibody testing *(14)* which should be read in conjunction with the current document.

The need for to assess the commutability of a secondary standard will depend on the type of standard, its status and intended use. For example, commutability assessment will not be necessary for a secondary standard that is to be used in a single laboratory and/or for a single method. However, commutability assessment should be considered for a secondary standard intended for use in multiple laboratories covering more than one method type, which may be the case for some regional secondary standards.

B.2.2 Assessment of need and procurement of material

Although the purposes for which a secondary standard may be needed are likely to be the same as for a WHO BRM, secondary standards are more likely to be used for routine testing.

The purpose for which a material is required should be explained to the candidate supplier, which is usually a manufacturer. The principle of "like versus like" as described above in Part A also applies to secondary standards, and will extend to the WHO BRM as well as the samples for which the secondary standard is intended to be used.

Frequently, materials will be supplied as final containers, often closed with rubber or elastomer stoppers. In such cases, it is very important to ensure that the contents of the individual containers are homogeneous. In other cases, the regional or national control authority will have to distribute a bulk material into final containers, and this will require appropriate facilities or the delegation of this task to an appropriate body.

B.2.3 Distribution of bulk material and processing of final containers

For lyophilized reference materials, the specifications for precision of fill, and residual oxygen and moisture content should be sufficient to assure the suitability of the material for its intended purpose. In many cases, secondary standards will be used regularly and batches will need to be replaced more frequently than those of WHO BRM. Stoppered vials are acceptable in place of sealed glass ampoules as the expected lifetime of a batch will be shorter than the expected lifetime of a WHO BRM. However, it is essential that the stability of the secondary standard is assured over the expected lifetime of the batch. It is advisable to monitor stability through an appropriate programme. Where available, a lyophilized, stable WHO BRM for the relevant measurand should be used as part of the stability programme.

B.2.4 Characterization and calibration

The characterization and calibration of a secondary standard is a complex process and specific guidance is provided in WHO manuals for different types of secondary standards *(12–14)* addressing the following aspects:

- the higher order reference material to which the secondary standards are traceable, which is usually the IS;

- compliance with regulatory requirements – the calibration of secondary standards for biological products should comply with local regulatory requirements, whereas the calibration of secondary standards for diagnostic use should follow the principles set out in ISO 17511 *(29)*;
- the anticipated use of the secondary standard and choice of assays used for the calibration exercise;
- parallelism or similarity of dose–response curves between the secondary and primary standard;
- whether a measurement uncertainty value should be assigned to the secondary standard – exemptions are described in ISO 17034 (subclause 7.13) for preparations not defined as CRMs *(48)*;
- considerations regarding the number of independent calibration runs to be performed in order to minimize the uncertainty associated with the assigned value;
- considerations for the assessment of commutability (for secondary standards with diagnostic application); and
- considerations for assessment of stability:

> For stability assessment, a representative biological product may be chosen as a further comparator. The specific activity of a commercial biological product is expected to remain relatively stable throughout its life-cycle, and can therefore be used to investigate any potential drift or shift of an in-house standard calibrated/monitored against the IS and its replacements.

Where a collaborative study is required (for example, to calibrate a regional or national standard), the number and geographical distribution of study participants is likely to be more restricted than for a global collaborative study to establish a WHO BRM. In some cases, only one or two participants (for example, the body intending to establish the secondary standard and/or the provider of the material) may be required. Great care should be taken to calibrate secondary standards as accurately as possible to avoid systematic bias in the estimation of activity. This may require a large number of independent assay runs. More specific recommendations in this regard are provided in the WHO manuals for secondary standards *(12–14)*.

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The resulting document was then posted on the WHO Biologicals website for a first round of public consultation in May and June 2025. Comments were received from: Dr L. Deprez, European Commission Joint Research Centre, Belgium; Dr A. Devonshire, National Measurement Laboratory, United Kingdom; Dr A. Griffin, National Association of Testing Authorities, Australia; Dr A. Ishii-Watabe, National Institute of Health Sciences, Japan; Dr Z. Liang and Dr Q. Mao, National Institutes for Food and Drug Control, China; Dr S. Morgeaux, Dr V. Vincent and Dr J. Korimbocus, Agence nationale de sécurité du médicament et des produits de santé, France; Dr H. Müller, Novo Nordisk Quality, Denmark; Dr M. Ochiai, National Institute of Infectious Diseases, Japan; Dr G. Praefcke, Paul-Ehrlich-Institut, Germany; Dr J. Southern, South African Health Products Regulatory Authority, South Africa; Dr R. Tierney, Dr G. Prescott and Dr B. Cowper, Medicines and Healthcare products Regulatory Agency, United Kingdom; and Dr S. Wendel, Hospital Sírio-Libanês, Brazil; Consolidated comments were received from: Dr M. Buda on behalf of the European Directorate for the Quality of Medicines & HealthCare, Council of Europe, France; Dr J. Joung, Dr C. Lee, Dr H. Oh, Dr N. Park, Dr S. Son and Dr E. Park on behalf of the Ministry of Food and Drug Safety, Republic of Korea; Dr J. Kress, Dr M. Prax, Dr G.H. Rubio Quintanares and Dr G. Unger on behalf of the WHO Collaborating Centre for Quality Assurance of Blood Products

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Taking into consideration the comments received from the first round of public consultation, the fourth draft document was prepared by Dr M. Nübling, consultant, Germany; Dr P. Stickings, Medicines and Healthcare products Regulatory Agency, United Kingdom; Mr P. Rigsby, Medicines and Healthcare products Regulatory Agency, United Kingdom; Dr P. Matejtschuk, Medicines and Healthcare products Regulatory Agency, United Kingdom; Dr M. Buda, European Directorate for the Quality of Medicines & HealthCare, Council of Europe, France; Dr T. Wu, Health Canada, Canada; Dr Q. Mao, National Institutes for Food and Drug Control, China; Dr G. Praefcke, Paul-Ehrlich-Institut, Germany; Ms C. Morris, Medicines and Healthcare products Regulatory Agency, United Kingdom; and Dr T.Q. Zhou, World Health Organization, Switzerland.

The fourth draft document was then reviewed during an informal consultation on WHO Recommendations for the preparation, characterization, establishment and use of WHO biological reference preparations, held virtually on 22–24 September 2025 and attended by: Dr Z. Ul Abidin, Drug Regulatory Authority of Pakistan, Pakistan; Dr M.A. Alharbi, Saudi Food and Drug Authority, Saudi Arabia; Dr M. Buda, European Directorate for the Quality of Medicines & HealthCare, Council of Europe, France; Dr N. Almond, Dr P. Bowyer, Dr C. Burns, Dr B. Cowper, Dr P. Matejtschuk, Ms C. Morris, Mr P. Rigsby and Dr P. Stickings, Medicines and Healthcare products Regulatory Agency, United Kingdom; Dr I. Feavers, consultant, United Kingdom; Dr M. Florens and Dr F. Ribaucour, Sciensano, Belgium; Dr J. Korimbocus, Agence nationale de sécurité du médicament et des produits de santé, France; Dr Q. Mao, National Institutes for Food and Drug Control, China; Dr Q. Meyer, National Control Laboratory for Biological Products, South Africa; Dr W.C. Moreira and Dr W.C. de Moura, National Institute for Quality Control in Health, Brazil; Dr K. Nguyen Thi, Ministry of Health, Viet Nam; Dr M. Nübling, consultant, Germany; Dr G. Praefcke and Dr A. Reissinger, Paul-Ehrlich-Institut, Germany; Dr M. Ochiai, National Institute of Infectious Diseases, Japan; Dr H. Oh, Ministry of Food and Drug Safety, Republic of Korea; Ms R. Pujilestari, Indonesian Food and Drug Authority, Indonesia; Dr S. Sebai Ben Amor, National Drug Control Laboratory, Tunisia; Dr T. Waddell, consultant, United Kingdom; Dr W. Wongchana, Ministry of Public Health, Thailand; and Dr T. Wu, Health Canada, Canada. *Representatives of the Developing Countries Vaccine Manufacturers Network (DCVMN)*: Ms V.L. Medica, PT Biofarma, Indonesia; Dr B. Ganneru, Bharat Biotech, India; Dr S.B. Reddy, GCBC Vaccines Pvt. Ltd., India. *Representatives of the International Federation of Pharmaceutical Manufacturers & Associations (IFPMA)*: Dr K. Aiyer, Teva Pharmaceutical Industries Ltd., USA. *Representatives of other entities*: Dr D. Smith, International Alliance of Biological Standardization, Canada; Dr L. Deprez, European Commission Joint Research Centre, Belgium; Dr C. Thelwell, International Society on Thrombosis and Haemostasis, United Kingdom; Dr Y. Nakagawa, Pharmaceutical and Medical Device Regulatory Science Society of Japan, Japan. *WHO Secretariat*: Dr T.Q. Zhou, Dr I. Knezevic, Dr Y. Maryuningsih, Dr D.

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Appendix 1

Information to be provided to WHO for the establishment of WHO biological reference materials

The following information should be provided by the coordinating laboratory in the initial summary report of the collaborative study. Following its submission to WHO, this report will become the BS summary report used to support a proposal to establish a WHO BRM. Not every aspect listed below will apply to all proposed WHO BRM.

1. Authors and summary

All report authors and their affiliations should be listed on the front page, with the project leader identified and their contact details provided. In the summary, the key aspects of the standardization project should be briefly described, the collaborative study findings summarized and the resulting proposal(s) clearly set out.

2. Introduction

The Introduction should explain in more detail the background and need for the proposed WHO BRM and should include:

- the name of the substance for which a WHO BRM is being proposed;
- a definition of the measurand;
- the rationale for the choice of proposed unit(s), where applicable;
- the rationale for the approach taken to ensure continuity of the unit, where applicable;
- the way in which the collaborative study was designed to evaluate the fitness for purpose of the candidate WHO BRM, including, where applicable, an assessment of its commutability;
- whether the candidate WHO BRM is needed to standardize products intended for the prevention and/or treatment of a disease or for its diagnosis/treatment monitoring;
- whether the reference material is subject to requirements for the manufacture and control of biological substances, is the subject of a monograph in a pharmacopoeia and/or is traded internationally;
- any recommendation by WHO or a recognized scientific organization that the reference material should be established;
- a review of methods currently used to assay similar materials, and the rationale for the choice of methods included in the collaborative study;
- the aims of the collaborative study and details of the participants;
- where a pilot study was performed, details of the material(s) used and results obtained; and
- if the reference material is intended for use in a diagnostic application, the approach used compared against the principles set out in ISO 17511, where applicable.

3. Bulk material

The following information should be provided:

- description of the bulk material, including its source, nature (including information on the donors(s) if relevant) and, where appropriate, its composition – this information may be supplemented by scientific literature references, patent information and/or package inserts;
- details and results of safety and other chemical, physical and biological testing performed, including testing of diluents where applicable;
- whether batches of bulk material were combined and, if so, the procedure used; and
- the composition (formulation) of the filled candidate WHO BRM, including buffers, diluents, bulking agents or stabilizers.

4. Processing of the candidate WHO BRM

The information provided should include:

- the identifying code of the candidate WHO BRM;
- the address of the facility where the bulk material was processed into final containers – where subcontractors were used for any processing stage, the identity of the subcontractor(s) should be provided, together with a list of the operations carried out by them;
- summary of the processing operations (filling, lyophilization and sealing) and the dates on which they were performed;
- the approach used to assess the precision of fill, together with the results obtained;
- the gas under which the material was sealed, its purity, the method used to determine the residual oxygen content in the containers (as an indicator of container integrity) 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);
- the approach used to assess the homogeneity of the candidate WHO BRM batch, together with the results obtained;
- a summary table of the characteristics of the candidate WHO BRM that includes, where applicable, target and obtained values for precision of fill, residual moisture and oxygen headspace – where target values have not been met, consideration of the fitness for purpose of the candidate WHO BRM should be included in the discussion section of the report. A suggested template summary table is provided below (see Table A1.1);
- details and results of any other testing performed on the contents of the final containers;
- the number of final filled containers in the batch available for use as a WHO BRM;
- the address of the intended place of storage and the name of the present custodian laboratory; and
- the recommended storage conditions, including temperature.

5. Stability

The information provided should include:

- the number of laboratories involved in obtaining the stability data, and details of the assay method(s) used;
- details of the stability study, including the number of assays carried out, details of the samples assayed, the temperatures and storage duration used, and estimates of the relative activity remaining in each container after exposure to each temperature (together with the corresponding 95% confidence intervals);
- an assessment of the stability of the candidate WHO BRM; and
- an assessment of the stability of the reconstituted candidate WHO BRM.

6. Collaborative study

The following information should be provided:

- general details of the study participants (for example, whether they are academic, industry or public health laboratories) and their geographical distribution – where the geographical distribution, and thus regional representativeness, of study participants is limited, a rationale should be provided and any constraints faced in ensuring broad regional representativeness among study participants highlighted;
- a statement in the relevant opening section to the effect that all study participants are denoted by a randomly allocated code number or letter in the main section of the report – all participants should be listed in full in Appendix 1 of the report (for example, alphabetically by country) but it must be expressly stated that the randomly allocated code numbers do not reflect the order presented in Appendix 1.
- the planning and design of the study, and descriptions of all materials included in it;
- the assay methods used and by how many participants – in order to ensure that study participants are not specifically identifiable in this respect, only their assigned codes should be used for this and subsequent reporting purposes;
- for each assay method, the number of runs that each participant was asked to perform and the number actually carried out;
- a description of all the statistical analysis methods used, including the way in which assay validity (system and sample suitability) was assessed and any problems that arose;
- results obtained from the statistical analysis, which can include:
    - the numbers of valid and invalid test results,
    - details of any exclusions (for example, of samples exhibiting non-similarity, or outliers) together with their justification and impact assessment,
    - comparisons of the results obtained using different assay methods, together with their interpretation and comments on particular aspects (such as the frequency distribution of the estimates, differences in estimates and any observed factors which may account for these),
    - for each assay method, the intra-laboratory variation and the overall inter-laboratory variation, and
    - the overall estimates obtained using each assay method:

        The (raw) data should be available on request to WHO (Secretary, ECBS) for a period of at least 20 years – or longer if the WHO BRM is still in use;

- for WHO BRM with a diagnostic application, conclusions regarding its commutability, indicating to which assay methods the conclusions apply and, where

appropriate, to what range of measurand concentrations – the commutability criteria used and how they were derived should also be clearly described; and

- for replacement WHO BRM, the final figures for the overall estimates of the proposed assigned value and their basis, together with details of any excluded data.

7. Other information

The report should also include:

- a proposal for the establishment of the candidate WHO BRM as a WHO BRM, together with an indication of any limitations on its use (for example, suitability only for certain assay methods) and a recommendation for the value(s) to be assigned, where applicable;
- for a proposed replacement IS, a consideration of the relationship of the unit that would be established by the replacement IS to that of the current IS, including evaluation of the extent to which continuity of the IU would be maintained;
- information on the uncertainty of content derived from the variance of the fill;
- uncertainty budgets and calculations for proposed WHO BRM with assigned SI units, as well as a description of the calibration hierarchy used for their value assignment;
- tables and figures showing the results of the collaborative study;
- closing conclusions and proposal(s), acknowledgements and references;
- a summary of participant comments received on the report;
- a copy of the proposed IFU containing the required information set out in Appendix 3 below;
- if requested by WHO or the ECBS, the detailed manufacturing records, including the results of in-process controls; and
- if requested, detailed results of tests performed on the bulk and/or filled material.

Table A1.1

Summary characteristics of candidate WHO BRM

General material characteristics		
Product code		
Presentation (liquid or lyophilized)		
Appearance		
Container type		
Number of containers available for distribution		
Date of fill		
Storage temperature		
Microbiological findings		
	Target value (where applicable)	**Result**
All presentations		
Mean fill mass (CV%)[a]		
Additionally for lyophilized material		
Mean dry weight (CV%)		
Mean residual moisture (CV%)		
Mean oxygen headspace (CV%)		

[a] CV% = coefficient of variation (%)

Appendix 2

Nomenclature of WHO biological reference materials

1. General principles

WHO BRM comprise the following categories:

- WHO International Standard (IS)
- WHO International Reference Panel (IRP)
- WHO International Reference Reagent (IRR)
- WHO Repository.

In the case of IS, IRP and repositories, an ordinal (such as “First”, “Second” etc.) is assigned to clearly indicate whether the material is the first such established IS, IRP or repository or a replacement. Replacement reference materials are typically established following depletion of the previously established IS, IRP or repository.

Examples of ordinal use include:

- First WHO International Standard for golimumab.
- Second WHO International Standard for alpha-fetoprotein (human).
- Fifth WHO International Standard for hepatitis B virus DNA for NAT-based assays.
- First WHO International Reference Panel for Lassa virus RNA for NAT-based assays.
- First WHO Repository of red blood cell transfusion relevant bacterial reference strains.

In the case of an IRP or repository, the option to subsequently add further individual members is available as an alternative to complete replacement.

Examples of panel member additions include:

- Expansion of the First WHO International Reference Panel for antibodies to SARS-CoV-2 variants of concern.

No ordinal is assigned to IRR.

Examples of IRR include:

- WHO International Reference Reagent for diphtheria antitoxin for use in flocculation test (equine).

The measurand(s) represented by a WHO BRM should be clearly and unambiguously named in line with common practice in the respective field of application. Common and widely used abbreviations in the names of measurands can be used (for example, DNA, RNA etc.). Less commonly used abbreviations should be avoided in the name of the WHO BRM and the respective terms spelled out in full. Consideration should be given to facilitating easy

identification of the measurand and associated WHO BRM by potential users performing electronic searches on websites, including the websites of WHO custodian laboratories and the online WHO BRM catalogue.[5]

The use of the above hierarchy and nomenclature conventions has proved highly useful, particularly in cases where an IS has been replaced on a frequent basis, and allows for the unambiguous identification in the scientific literature of the precise WHO BRM relevant to the published results. As part of ongoing nomenclature harmonization in this area, the naming of all new WHO BRM should be aligned with the WHO naming conventions in place at the time. This should also be the case for replacement WHO BRM names, which as a result may on occasion depart from the naming convention used for the WHO BRM being replaced, unless, and on a case-by-case basis, retaining the naming convention previously used can be justified.

2. WHO BRM for polyclonal antibodies

To promote consistency in the naming of WHO BRM for polyclonal antibodies, the following template should wherever possible be used in the case of antibodies against a specified antigen or pathogen:

[First, Second etc.] WHO International Standard [or WHO International Reference Panel] or [No ordinal] WHO International Reference Reagent] for antibodies to [antigen or pathogen] for [assay category].

Where required for clarity, additional qualifiers (for example, “human serum”, “human immunoglobulin”, “human plasma”) should be used consistently and be placed in parentheses at the end of the WHO BRM name.

Examples of names for WHO BRM for polyclonal antibodies include:

- Third WHO International Standard for antibodies to rabies virus.
- First WHO International Standard for antibodies to citrullinated peptide/protein.
- First WHO International Standard for antibodies to Nipah virus for binding assays (human serum).
- WHO International Reference Reagent for antibodies to Ross River virus for neutralization assays (human plasma).
- First WHO International Reference Panel for antibodies to SARS-CoV-2 variants of concern.

3. Assay-specific WHO BRM

Where a WHO BRM has been recommended by the ECBS for use in a specific assay type only, this should be indicated in the name.

Examples of assay-specific WHO BRM include:

[5] Available at: https://www.who.int/teams/health-product-policy-and-standards/standards-and-specifications/norms-and-standards/catalogue.

- WHO International Reference Reagent for the quantitation of lentiviral vector copy number by quantitative PCR.
- WHO International Reference Reagent for the quantitation of lentiviral vector copy number by digital PCR.
- Fifth WHO International Standard for hepatitis B virus DNA for NAT-based assays.
- First WHO International Standard for antibodies to Rift Valley fever virus for neutralization assays (human plasma).
- First WHO International Standard for antibodies to Rift Valley fever virus for binding assays (human plasma).

4. Use of qualifiers

Qualifiers should be used sparingly and appear in parentheses at the end of a WHO BRM name, and only in cases where ambiguity could otherwise potentially exist. For example, where a native WHO BRM has been replaced by a recombinant material, the recombinant nature of the new WHO BRM should be indicated.

Examples of required qualifier use include:

- Third WHO International Standard for interferon B (human, recombinant, glycosylated).

The qualifier "(human)" is generally only required where corresponding non-human WHO BRM for use in standardizing human biological products and associated processes have been, or potentially may be, established.

5. Use of international nonproprietary names

An international nonproprietary name (INN) may be in existence for a material for which a WHO BRM is established. However, unless the WHO BRM is intended to be used to standardize only that material complying with the definition of the INN, the INN is not included in the name of the WHO BRM, but should be included in the accompanying IFU.

Examples of appropriate non-INN use include:

- Third WHO International Standard for tissue plasminogen activator (human, recombinant).

In the above example, although a preparation of recombinant tissue plasminogen activator has been assigned the INN "Alteplase", the WHO BRM (prepared from Alteplase) is intended for use in standardizing assays of tissue plasminogen activator from all sources.

Conversely, for antibiotics and biotherapeutic products such as monoclonal antibodies – where the assignment of an INN is an inherent part of product development – the use of the INN is unavoidable.

Examples of appropriate INN use include:

- Third WHO International Standard for neomycin.
- First WHO International Standard for cetuximab.

- First WHO International Standard for golimumab.

Appendix 3

Information to be included in the instructions for use for users of WHO biological reference materials

The instructions for use (IFU) provide important information about the WHO BRM, including a description of its intended use(s), contents, assigned value(s), stability, and other relevant supporting information or references. A reference to the respective BS summary report should always be included, where available. The IFU should also, where possible, contain a copy of the product label, since this can facilitate the export and import of the WHO BRM. The name of the WHO BRM on the label must match the name given in the IFU to avoid potential delays at customs when shipped.

The IFU for each WHO BRM should accompany every shipment and also be made available via the website of the custodian laboratory. A template for the contents of an IFU is shown in Table A2.1 below. In addition, every IFU must also clearly state:

- the name of the WHO BRM, along with its category, identifying code and year of establishment; and
- the version number of the IFU and date issued.

Table A2.1

Summary contents of an IFU[a]

IFU section	Content considerations
1. Intended use	*Short description of the intended use(s) (for example, calibration of secondary standards, initial validation of assays or regulatory check of assays); information on potential limitation of uses; summary of results of commutability evaluation where applicable; reference to the citation of the BS summary report in section 9 below.*
2. Caution	*Opening statement, in bold and underlined, to the effect that "This material is not intended for administration to humans or to animals in the food chain"; details on the origin of the material, health status of donors, testing/screening and inactivation steps performed, and potential infectivity; recommended handling of the material, including precautionary measures to be taken.*
3. Unitage	*Assigned value(s) or other parameter; information on uncertainty, where applicable.*
4. Contents	*Details of the nature and formulation of the filled material and its country of origin; mean fill volume or mass.*

5. Storage	*recommended storage conditions, including storage temperature.*
6. Directions for opening	*Instructions for opening, with cautionary information included where necessary.*
7. Use of material	*Method of reconstitution if applicable (including recommended reconstitution volume and procedure); period of use, storage conditions after reconstitution and proposed dilution matrix/matrices; directions for safe use and disposal before and after reconstitution.*
8. Stability	*Information on stability and explanation that the material may be shipped at a higher temperature compared to the recommended storage temperature where this is supported by evidence from stability studies.*
9. References	*Citation of the BS summary report presented to the ECBS and any additional scientific publications relevant to the development or use of the WHO BRM; other WHO manuals or guidance documents as appropriate.*
10. Acknowledgements	*Typically includes collaborative study participants, providers of source material and advisors.*
11. Further information	*May include reference to relevant WHO online resources for biological products, such as the WHO BRM catalogue, and to the custodian laboratory and other relevant entity websites.*
12. Customer feedback	*Expressing encouragement for customer feedback, suggestions and complaints through the feedback email address provided.*
13. Citation	*Instructions for the full and correct citation of the WHO BRM and provider custodian laboratory details.*
14. Liability and loss	*Disclaimer concerning liability and other terms and conditions with regard to the use of the material.*
15. Information for customs use only	*Indicates country of origin, net weight, toxicity status and inclusion of veterinary certificate or other statement where applicable.*
16. Certificate of Analysis	*Includes explanation of why a Certificate of Analysis is not provided for WHO BRM and explicitly states that all such reference materials are established in accordance with this current WHO Recommendations document, which should be cited and a link provided.*

[a] In addition to the IFU, a **Materials Safety Data Sheet** should also be provided that clearly shows the key physical, chemical and toxicological properties of the WHO BRM, along with first aid advice and actions to be taken in the event of spillage.