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# WHO manual for the preparation of reference materials for use as secondary standards in antibody testing

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# Annex 2

## WHO manual for the preparation of reference materials for use as secondary standards in antibody testing

Guidance documents published by the World Health Organization (WHO) are intended to be scientific and advisory in nature. Each of the following sections constitutes guidance for national regulatory authorities (NRAs) and for manufacturers of biological products.

## Abbreviations

| | |
|---|---|
| BSL | biosafety level |
| COVID-19 | coronavirus disease 2019 |
| ELISA | enzyme-linked immunosorbent assay |
| FRNT | foci reduction neutralization test |
| GMP | good manufacturing practice(s) |
| HPV | human papillomavirus |
| IFU | Instructions for Use |
| IS | International Standard(s) |
| IU | International Unit(s) |
| MSC | microbiological safety cabinet |
| MTA | material transfer agreement |
| MU | measurement uncertainty |
| PRNT | plaque reduction neutralization test |
| PV | pseudotyped virus |
| QC | quality control |
| RBD | receptor binding domain |
| RSV | respiratory syncytial virus |
| SARS-CoV-2 | severe acute respiratory syndrome coronavirus 2 |
| SI | International System of Units |
| SOP | standard operating procedure |

## 1. Introduction

The development, establishment and promotion of international reference standards for biological materials is a core function of WHO and plays an important role in ensuring the quality and consistent dosing of biological medicinal products used worldwide. These standards are widely used in the development, evaluation, standardization and control of such products by industry and regulatory authorities, as well as supporting biological research in other scientific organizations.

WHO International Standards (IS) are established by the Expert Committee on Biological Standardization with an assigned International Unit (IU). Metrologically, IS serve as the primary standard for the calibration of national and other secondary standards, and are considered to be of the highest order. Consequently, it is important to conserve the typically limited stocks of an IS, and to this end national authorities frequently consider establishing their own secondary reference materials (see Appendices 1–4). Similarly, manufacturers or research centres conducting numerous assays as part of their product development programme usually establish a secondary standard for routine use. The biological activities of such secondary materials should be calibrated in IU by direct comparison with the respective IS.

The WHO Recommendations for the preparation, characterization and establishment of international and other biological reference standards was adopted in 1978 and was most recently revised in 2004 *(1)*. Subsequent feedback from national control laboratories (NCLs), vaccine manufacturers and diagnostics producers led to the publication of two WHO manuals to address practical issues in the establishment of national and secondary standards for: (a) vaccines *(2)*; and (b) in vitro diagnostic assays for infectious diseases based on nucleic acid or antigen detection *(3)*.

The coronavirus disease 2019 (COVID-19) pandemic has led to a major global effort to develop vaccines and therapeutics, including antibody-based therapeutics. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the etiological agent of COVID-19 and causes mild or asymptomatic infection in the majority of cases; however, around 10% of cases require medical intervention and a small proportion result in severe pneumonia and death. In 2020, the First WHO International Standard for anti-SARS-CoV-2 immunoglobulin was established to facilitate the development and harmonization of serological assays to a common unitage *(4)*. These assays provide information on potential immune correlates of protection and are essential in supporting the clinical development of vaccines and therapeutics, as well as the seroepidemiological studies required to assess the impact of COVID-19. The assays broadly fall into two categories – virus neutralization assays and antibody binding assays such as enzyme-linked immunosorbent assays (ELISAs) (see Appendix 5). Plaque or foci reduction neutralization tests (PRNTs or FRNTs respectively) and microneutralization assays (see Appendix 6) are widely regarded as the reference methods for measuring potentially protective antibodies against many viral diseases. Such assays involve the use of live virus, which in the case of SARS-CoV-2 requires laboratories at biosafety level 3 (BSL3). However, the use of pseudotyped viruses (PVs) in neutralization assays (see Appendix 7) has been shown to be a potential alternative, including systems based on lentiviral and varicella zoster virus PVs widely used for detecting neutralizing antibody to SARS-CoV-2 *(5, 6)*. In addition to these virus neutralization assays, other functional assays for anti-SARS-CoV-2 antibodies include, but are not limited to, assays that measure antibodies that block the viral receptor binding domain (RBD) from binding to the ACE-2 receptor and antibody-dependent cellular cytotoxicity assays.

Current human papillomavirus (HPV) vaccines are based on virus-like particles consisting of recombinant capsid proteins. The standardization of assays for HPV capsid

antibody (see Appendix 9) has supported vaccine development and continues to underpin epidemiological studies. In recent years, WHO IS for HPV antibodies have been established for virus serotypes 16 and 18.

Respiratory syncytial virus (RSV) is a significant cause of lower respiratory illness in infants, the elderly and immunocompromised individuals, and the development of a vaccine remains a global priority. Activity in this area has increased in recent years, and in 2017 the First WHO International Standard for antiserum to respiratory syncytial virus was established (see Appendix 10). Initially recommended for use in the assessment of RSV subtype A (RSV/A) neutralization titres in human serum, the standard was extended to include subtype B (RSV/B) in 2019.

Worldwide demand for the anti-SARS-CoV-2 WHO IS and for many other antibody standards (for example, for HPV and RSV) has inevitably led to the development of national and other secondary reference materials. Thus, in addition to the WHO manuals on secondary standards for vaccines and in vitro diagnostics that rely on nucleic acid or antigenic components for virus detection, the increasing demand for antibody standards has highlighted the need for the current WHO manual on the calibration of secondary standards for the evaluation of antibody responses to infection and vaccination.

## 2. Purpose and scope

Antibody reference materials are used to minimize the inherent variability across different assays used to evaluate antibody responses, and to ensure uniformity in the designation of potency or activity to immune sera and potentially therapeutic antibody preparations. The term “secondary standard” as used in this document includes all such reference materials developed by regional or national authorities, manufacturers and others and calibrated against the WHO IS. Such secondary standards are intended to provide greater quantities of calibrated material than would otherwise be available from the limited supply of the IS.

The principal focus of this document is on the preparation of secondary standards for use in evaluating antibody responses elicited either by natural infection or vaccination. Such standards may also be used to ensure the consistent dosing of human convalescent plasma and monoclonal antibodies in the treatment of infection, and to qualify or validate in vitro diagnostics (IVDs) and other test procedures based on antibody detection. However, the qualification or validation of serological test procedures is typically achieved using panels of low-, medium- and high-titre sera calibrated against the WHO IS and is beyond the scope of this document.

Although the current document and several of its appendices focus on the development and calibration of secondary standards for the evaluation of antibody responses to SARS-CoV-2, it is not limited to SARS-CoV-2, with many of the principles set out having been derived from the development of antibody standards for other infections. The manual is therefore suitable for laboratories wishing to establish secondary standards for use in evaluating antibody responses to any infectious agent. Furthermore, the document provides general guidance on the principles of the preparation of secondary standards for use in antibody testing. Specific issues associated with the preparation and recommended application of any particular standard must be considered on a case-by-case basis.

The document is intended for use by NCLs and other laboratories requiring reference materials for antibody-based disease assays, manufacturers of secondary standards, manufacturers of vaccines and antibody-based assays (including antibody-based IVDs) and providers of external quality assurance schemes. The document supplements the guidance provided in the WHO Recommendations for the preparation, characterization and establishment of international and other biological reference standards *(1)*. Analogous

guidance has been published by WHO on the preparation of secondary standards for vaccines *(2)* and for IVDs for infectious diseases based on nucleic acid or antigen detection *(3)*.

## 3. Terminology

The definitions given below apply to the terms as used in this WHO guidance document. These terms may have different or broader 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 the bioassay.

**Antibody binding assay**: a bioassay that measures antibody binding to its target antigen.

**Antiserum**: blood serum that contains antibodies against a specific infectious agent.

**Assay**: a measurement procedure – that is, a detailed description of a measurement according to one or more measurement principles and to a given measurement method, based on a measurement model and including any calculation needed to obtain a measurement result.

**Baseline parameters**: the optimal storage conditions for maintaining the biological and/or immunological activity of a biological material, and which are used for comparative purposes against other storage conditions.

**Calibration**: an operation that, under specified conditions, in a first step, establishes a relation between the quantity values with measurement uncertainties provided by measurement standards and corresponding indications with associated measurement uncertainties and, in a second step, uses this information to establish a relation for obtaining a measurement result from an indication.

**Calibration hierarchy**: a sequence of calibrations from a reference to the final measuring system, where the outcome of each calibration depends on the outcome of the previous calibration *(7)*.

**Calibrator**: a calibration material used to adjust the output from a measuring system based on, or traceable to, a reference material preparation.

**Certified reference material**: a reference material accompanied by documentation issued by an authoritative body and providing one or more specified property values with associated uncertainties and traceability, using valid procedures.

**Commutability**: the property of a reference material demonstrated by the closeness of agreement between the relation among the measurement results for a stated quality in this material, obtained according to two measurement procedures, and the relation obtained among the measurement results for other specified materials *(8)*.

**Custodian laboratory**: the institute or other entity responsible for developing, storing and distributing a given standard.

**Dose response**: the relationship between the amount of a material and its biological effect.

**Functional antibody assay**: a bioassay that measures the biological and/or immunological activity of an antibody that reduces disease (for example, neutralizing, opsonophagocytic or complement-mediated activity).

**Immunoassay**: an immunological test procedure that uses antibodies to measure an **analyte** in a biological sample.

**Independent assays**: mutually exclusive test procedures.

**International biological measurement standard**: a **certified reference material** (referred to as a WHO IS) derived from a biological substance (that is, one that cannot be fully characterized by physicochemical means alone and is measured using a bioassay) and

which enables the results of biological or immunological assays to be expressed in the same way worldwide.

**International Unit (IU)**: the unitage assigned by WHO to an **international biological measurement standard**.

**Linearity**: the ability to provide laboratory test results that are directly proportional to the concentration of the **measurand** in a test sample.

**Measurand**: the quantity of **analyte** intended to be measured in an assay.

**Methodology**: the specific procedures or techniques used to analyse a material.

**Neutralizing antibody**: an antibody that renders a virus non-infectious or a toxin ineffective.

**Plasma**: the liquid component of blood from which the blood cells have been removed but retaining clotting factors and proteins, including antibodies.

**Platform**: a technology or group of technologies that form the basis of an analytical process.

**Potency**: an expression of the activity of a biological material in terms of the amount required to produce a defined effect.

**Precision**: the closeness of agreement between indications or measured quantity values obtained by replicate measurements on the same or similar objects under specified conditions.

**Primary standard**: a **certified reference material** commonly referred to as a WHO IS.

**Reference standard**: a measurement standard designated for the calibration of other measurement standards that provides a consistent basis for the measurement of quantity or potency.

**Secondary (reference) standard**: a **reference standard** calibrated against (and traceable to) a WHO IS, and established by regional or national authorities, or by other laboratories.

**Specimen**: a discrete portion of a body fluid or tissue taken for examination, study or analysis of one or more quantities or characteristics to determine the character of the whole.

**Tertiary (reference) standard**: a reference material, such as a working reagent or standard, product calibrator or control material, calibrated against the **secondary (reference) standard**.

**Test**: an in vitro assay for a specific **analyte**, including the instrument(s) used.

**Traceability**: the metrological property of the result of a measurement or the value of a standard whereby it can be related to stated references, usually national or international standards, through an unbroken chain of comparisons, all of which have stated uncertainties.

**Uncertainty**: an estimate attached to a test result or a higher-order reference material (calibrator) that characterizes the range of values within which the true value is asserted to lie with a stated probability.

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

**Working standard**: a measurement standard used routinely to calibrate or verify measuring instruments or measuring systems for a specific assay.

## 4. Using biological standards

The purpose of metrological traceability is to ensure that a measurement takes into account all uncertainties and is an accurate representation of the material being measured. Thus, the results of an assay should be expressed in terms of the values obtained at the highest level of the calibration hierarchy *(7)* – which in the physical sciences means obtaining values in the

International System of Units (SI). However, it is difficult to assign a value unambiguously in SI units to the biological activity of a complex analyte such as an antibody or immune serum. Instead, arbitrary units are assigned to the biological activity of the material by measuring its potency relative to an established reference standard. The approach taken by WHO to measuring biological activity is to establish the highest order reference standard (the WHO IS) with a value assigned in IU. Other lower-order biological reference materials for a given analyte can then be related through a sequence of comparisons traceable to the IS.

As the highest order biological reference standard, it is vital to maintain stocks of the IS, which are typically available in limited quantities and are a finite resource. Although WHO recommendations provide for the replacement of IS, frequent replacement increases the risk of the assigned unitage drifting over time. Therefore, secondary standards, calibrated directly against the IS, should be established for use in the calibration of tertiary or working standards and for the initial validation of new assays. Regional or national reference materials are usually secondary standards. In addition, manufacturers and research laboratories performing large numbers of assays may develop secondary standards calibrated directly against the IS. To conserve supplies of the IS, it should not be used by manufacturers as an in-house standard, a run control, a working standard or a calibrator. Table1 summarizes the key properties of WHO IS, secondary standards and tertiary standards.

**Table 1**
**Key properties of WHO IS, secondary standards and tertiary standards**

| Property | WHO IS | Secondary standard | Tertiary standard |
|---|---|---|---|
| **Alternative names** | Highest order international conventional calibrator | Regional or national reference material or standard | Working reagent or standard; internal assay reference reagent |
| **Calibration** | Evaluated in an international collaborative study involving laboratories worldwide, different assays and different types of laboratories (usually 15–30 participants) | Calibrated against the WHO IS | Calibrated against the secondary standard |
| **Unitage** | IU/mL | IU/mL | IU/mL |
| **Traceability** | N/A | Yes | Yes |
| **Uncertainty of measurement** | No | Yes (assay specific) | Yes (assay specific) |
| **Commutability** | Must be determined experimentally relative to clinical specimens | Should be determined experimentally relative to clinical specimens | Consideration should be given to experimentally determining relative to clinical specimens |
| **Material** | Should resemble, as closely and feasibly as possible, the analyte being measured – for | Should resemble, as closely as possible, the analyte to be measured. However, for assay-specific | Should resemble, as closely as possible, the analyte to be measured. Biological material similar to the |

| | | | |
|---|---|---|---|
| | example, for SARS-CoV-2 antibody standards, natural samples from SARS-CoV-2 recovered or vaccinated individuals | secondary standards, recombinant antibodies or animal serum may be used, with laboratories encouraged to address commutability | tested sample (such as recombinant antibodies or animal serum) may be used, with laboratories encouraged to address commutability |
| **Typical final format of standard** | Lyophilized | Lyophilized, liquid or dry tube specimen | Liquid or dry tube specimen |
| **Usage** | Calibration of secondary standards; initial validation of new assay/platform | Calibration of tertiary standards; working standards; run controls; and calibrators | Working standards; run controls; calibrators |
| **Establishment of standard** | International agreement through a WHO international collaborative study, proposal for adoption and subsequent establishment on the recommendation of the WHO Expert Committee on Biological Standardization | May be calibrated in several ways:<br><br>1. In parallel with a study to establish the IS<br><br>2. Regional or national collaborative study similar to the WHO collaborative study but with fewer participants<br><br>3. Small study by one or a limited number of laboratories with a single assay or a limited number of different assays/platforms (assay-specific secondary standards) | 1. Assay-specific study, normally by a single laboratory for use with a specific test/platform<br><br>2. Small study by a limited number of laboratories with a single assay or a limited number of different assays/platforms |

Although in general, antibody standards based on plasma or serum are relatively stable, reliance on the value assigned to any biological reference material depends upon its stability. Consideration should therefore be given to the stability of the reconstituted material both in storage and in use (see sections 12 and 13 below).

In many cases, the IS will not yet have been established at the time of early clinical studies and antibody assays will need to be standardized using an antiserum working reagent. This is particularly likely to be the case during public health emergencies (such as the COVID-19 pandemic) when vaccine and therapeutic antibody development proceeds at pace before sufficient convalescent serum is available to produce the IS. However, provided that sufficient working reagent is retained, the results of these early studies could be retrospectively converted into IU once the IS has been established.

## 5. Principles for preparing secondary standards for antibodies

Compared to other biological reference materials, a polyclonal antibody standard for a defined infectious agent is unique since a polyclonal serum or plasma contains different quantifiable analytes, with each of the analytes defined by both the antigen/epitope against which it is directed and its antibody class (for example, IgG, IgA or IgM). Dependant on assay design, assays to be harmonized by an antibody standard are either measuring a biological activity (such as neutralization capacity, as exhibited by subsets of antibodies) or are measuring binding antibodies as characterized by the target antigen(s) and antibody class(es) detected. Each analyte present in the standard and of relevance to the harmonization of the respective assays must be formally defined by unitage.

However, antibody standards also have certain essential characteristics in common with other biological standards and reference materials that are critical to their function:

- They consist of a single batch of identical containers.
- The characteristics of the standard should be comparable to those of the samples to be tested.
- They have a formally defined unitage, assigned using appropriately designed studies and assays.
- They are stable with respect to that formally defined unitage.

The requirements for establishing IS are detailed in the WHO Recommendations for the preparation, characterization and establishment of international and other biological reference standards *(1)*. In general, these high-level requirements are equally applicable to secondary standards but with key differences that may allow for some flexibility. Most notably, the IS is the highest order standard and is not, therefore, defined by any other external reference, whereas the value assigned to a secondary standard is defined in units traceable to the higher-order IS.

If more than one batch is prepared from the same bulk and assessed for suitability in a collaborative study and one batch is established as the standard, the other fills may be considered for establishment as secondary standards providing they are of sufficient quantity and stability to meet demand.

The calibration of a secondary reference material is a complex process and considerations that should be taken into account include:

- Traceability – the process by which the unitage of each analyte is assigned to the secondary standard relative to the IS is the “traceability path”, and should be clearly defined.
- Uncertainty – any formal definition of a secondary standard in terms of a higher-order standard (such as the IS) must include handling of uncertainty. Where several methods have been used to calibrate a secondary standard, it may not be valid to make assumptions across the methods with regard to a single underlying true value or a probability distribution of values to estimate uncertainty. In such cases, uncertainty will be assay specific.
- Value-assignment methodology – the traceability path and uncertainty are only valid for the assay methodologies used to assign the value of the standard. For some IS, units are assigned for specific assays (for example, in virus neutralization or enzyme immunoassays). In such situations, it will be necessary to value-assign a secondary standard using a specific assay method. In principle, it should not be

necessary to recalibrate existing secondary standards when the IS is replaced but the suitability of the replacement in this regard should be checked before it is established.

- Stability – the stability of a secondary standard is usually monitored in real time against the IS. Ideally this should be checked through ongoing monitoring of a suitable parameter appropriate for the assay used (such as neutralization titre).
- Commutability – commutability is the extent to which the reference material will be a suitable standard for all of the various types of samples being evaluated. When appropriate and feasible, commutability should be assessed as part of a collaborative study by including a panel of the different types of samples for which the standard will typically be used.

A procedure should be put in place for establishing and monitoring secondary standards, and for their holding and distribution, including the responsibilities of the custodian laboratory and any other bodies involved in the process.

IS are likely to be lyophilized to ensure their stability for many years. In contrast, secondary standards are used as working standards and, therefore, need to be formulated so that they are stable throughout the period of their use. Ideally standards are sterile – however, materials of low bioburden may be acceptable provided that this does not interfere with the assay, or affect standard stability or safety, and that the materials are kept under appropriate conditions to minimize potential bioburden.

The preparation and calibration of secondary standards requires a considerable amount of work and should not be undertaken lightly. Extensive experience and expertise are required, including appropriate statistical support, and training may be required. For these reasons, it is recommended that, whenever possible, countries collaborate in the development of regional standards to minimize duplication of effort.

## 6. Planning

The current document sets out the issues that must be considered in the preparation of secondary antibody standards. The laboratory producing a candidate secondary standard should take into account the intended use and demand so that the batch of standard will last at least 3–5 years. The laboratory should have access to appropriate filling and processing facilities, as well as adequate storage and distribution facilities.

The following issues should be considered prior to developing a secondary standard and it may be informative to survey likely users of the standard using a questionnaire formulated to gather the required information:

- What type of assay will the standard be used in? For example, antibody binding assays may require a smaller volume of standard than a functional assay such as those used to evaluate neutralization or opsonization, while some automated high-throughput assay systems may require a dead volume.
- Related to the point above, what would be the most appropriate fill volume and type of container?
- How many vials/ampoules will be used in each assay?
- How many vials/ampoules will be used annually by each user?
- Will the standard be suitable for single or multiple antibody specificities?
- What would be the ideal shelf-life of the proposed secondary standard?
- Is the material infectious and, if so, what precautions could be taken to mitigate any risk to users?

In light of such considerations, the likely annual demand can then be determined, and an appropriate volume of bulk material sourced and number of containers prepared. Planning should also take into account the number of containers that may be required for calibration and stability studies. Appendix 1 lists the documentation and records to be compiled during a standardization project.

Although serological standards are developed for a wide variety of assays, it is unlikely that the unitage assigned using one assay will be applicable to all assays because each assay is likely to detect different analytes. Careful consideration needs to be given to the choice of assay used in the development of the standard and subsequent recommendations provided on the type and design of assay in which the standard should be used.

## 7. Selection of candidate material

The characteristics of a secondary antibody standard should resemble as closely as possible those of the test samples in the assay systems in which the standard will be used. Thus, in the case of clinical vaccine trials, evaluations of convalescent serum, infection studies and seroepidemiological analyses the secondary antibody standard will typically be derived from a pool of human plasma or serum.

The pool may consist of plasma or serum from either convalescent or vaccinated individuals, depending on the intended application of the standard. The specificity of the antibodies in the standard will depend upon the source of the material and therefore needs careful consideration during project planning to ensure that the secondary standard resembles the test samples in the assay systems in which it will be used. In general, convalescent plasma or serum will have broader antibody specificity against an infectious agent than plasma or serum from vaccinees, which will contain antibodies specific for the vaccine antigen(s). For example, many COVID-19 vaccines are based on the S antigen and therefore anti-S antibodies will be predominant in plasma obtained from the vaccinees.

In addition, microbial pathogens are often antigenically diverse and can evolve novel variants of key antigens over time. In the case of SARS-CoV-2, for example, so called variants of concern, carrying mutations that render them more transmissible and/or resistant to acquired immunity, continue to emerge. This presents a challenge when sourcing candidate material for a secondary standard, which should be as similar as possible to the primary standard used to calibrate it. Any change in the source material potentially risks introducing a change in analyte(s) causing a shift in the unitage. Potential changes in the predominant variant and the vaccination status of prospective donors therefore need to be considered carefully when sourcing candidate material.

To ensure the safety of the standard, individual donations should be negative for known bloodborne virus markers (for example, of human immunodeficiency viruses and hepatitis viruses) and, if necessary, the treatment of candidate material using an appropriate validated method to reduce the risk of viral contamination should be considered. For example, the risk of the presence of enveloped viruses may be reduced using a solvent-detergent treatment. Consideration should be given to the potential impact of such treatment on the characteristics of the material in the assay systems in which it will be used.

Typically, the bulk material will be collected as part of a study at one institution before being transferred to one or more other laboratories for processing, storage and distribution. Given the potentially infectious nature of such standards, the use of a material transfer agreement (MTA) will ensure that known risks and mitigations are made clear to all parties during handling and transport. An MTA can also be used to ensure that all parties adhere to specific legal and ethical considerations relating to the material.

Sufficient volume of bulk material should be filled to ensure that the standard will last for at least 3–5 years. Although relatively large volumes of plasma may be obtained from healthy adult volunteers (for example, by plasmapheresis), this typically means that plasma donations from a number of individuals will be required. Sufficient time should be allowed following the onset of symptoms (or vaccination) for the antibody response to be induced. Individual donations should be characterized by a laboratory with experience of the immunoassays in which the standard will be used. Based on the resulting data, a decision can then be made on which donations to include in the final pool. When pooling individual donations, consideration should be given to the anticoagulant in each individual sample, and to ensuring that it has been validated for the assay(s) in which the secondary standard will be used. Pooling samples containing different anticoagulants is not recommended.

The pooling procedure should ensure that the material is mixed thoroughly and is homogeneous. Care should be taken to avoid the denaturation of protein during mixing. In addition to any studies of the individual batches before pooling, the homogeneous blend should also be characterized to demonstrate its suitability for use as a standard.

Ideally, individual plasma donations should be stored frozen below −70 °C until ready for pooling and filling. Careful planning will ensure that freeze-thawing is minimized. For example, samples can be taken from plasma donations for characterization prior to freezing and stored separately. Also, the bulk material can be pooled and filled into the final container on the same day to avoid refreezing the bulk pool. The containers used for storage should be able to withstand the freezing, storage and thawing conditions, and the storage conditions should ensure that the immunological properties of the material are conserved.

# 8. Processing of final container

## 8.1 Quality aspects

Although the manufacturing of reference standards does not require adherence to good manufacturing practices (GMP), it is important that the whole standard-preparation process be controlled and documented within the context of a quality system. All operators should be trained and key variables (reagents used, operating equipment, software, and process times and cycles) should be documented and any equipment used for manufacture or quality control (QC) testing must be kept in recordable certification. Once QC testing is available, the manufacturing process and QC testing results should be reviewed and approved before the standard is distributed.

***Note***: the specific examples of standard operating procedures (SOPs) provided in the Appendices 5–10 of this manual may indicate adherence to GMP in their jurisdiction but this is not a global requirement for the preparation of reference standards.

## 8.2 Nature of the secondary antibody standard

Antibody standards may be lyophilized, liquid or frozen liquid. They are generally lyophilized, as experience has shown this to be a consistently stable format that facilitates distribution. Although this is the preferred option, there may be circumstances in which the immunological characteristics of the standard would be affected by lyophilization or subsequent reconstitution of the material. If lyophilization is not possible or desirable, the distribution of frozen or liquid standards may be considered depending on the stability of the material. Stability should be determined by temperature-stressing studies. If the secondary

standard needs to be shipped under refrigerated conditions (2–8 °C) or as a frozen liquid, the cold chain during transportation should be validated. Repeated freeze-thawing of frozen standards should be avoided because of the potential impact on the stability of the material. To avoid unnecessary freeze-thawing, the fill volume should be considered carefully and an aliquoting strategy employed if freeze-thawing is absolutely necessary. Freeze-thaws, if any, should be documented and it should be demonstrated that this does not affect the activity of the material.

## 8.3 Container format

The choice of container should be evaluated during pilot studies and shown not to affect the characteristics of the standard. Studies have shown that reference standards stored in vials with elastomeric closures (such as rubber stoppers) may exhibit inferior storage stability compared to those supplied in flame-sealed glass ampoules (the preferred container for an IS). Vials with elastomeric closures are, however, more convenient and may be more suitable for secondary standards used in certain assay formats. The suitability of the rubber closures for the chosen storage conditions should be assessed as some formulations become brittle at low temperatures, compromising the integrity of the seal. Vials should be of good quality glass appropriate for pharmaceutical use. Plastic vials may be required in certain circumstances – for example, to meet biocontainment requirements – in which case they should also be of pharmaceutical quality.

## 8.4 Microbial bioburden

Ideally, standards should be sterile as microbial contamination may interfere with their performance in certain immunoassays. This may require particular consideration for cell-based assay systems (for example, virus neutralization or opsonophagocytosis assays) or where an assay requires the subsequent culturing of the infectious agent (for example, complement-mediated killing assays). Although strict sterility is not always required and may not be easily achieved in practice, it is advisable to minimize the risk of microbial contamination. This may be achieved by use of appropriate filling facilities with clean room technology applied to filling processes (including lyophilization where used) and appropriate personal protective equipment to minimize contamination of the material during filling and drying where applicable. Suitable environmental monitoring will be required, including particle and microbial monitoring of the process area, along with appropriate batch-testing of the candidate standard.

## 8.5 Accuracy/consistency of fill

The filling process should be well controlled so that the amount of active reference standard is within tightly defined limits and consistent across the batch. Although this limit may not need to be as tightly defined for secondary standards compared to an IS, it should still be appropriately controlled within a pre-defined range, and documented. The limit will reflect what is achievable by the filling equipment and the precision of the assays for which the standard is to be used. Typically, an IS is filled within a coefficient of variation of 0.25% and then lyophilized. This does not apply to liquid or frozen standards because reconstitution volume errors cannot occur and volumes can be measured accurately at the time of use.

## 8.6 Freeze-drying cycles

Lyophilization conditions should be based on the need to deliver stable standards of good and consistent quality. Sample formulations intended for lyophilization may be analysed by thermal analytical methods and/or freeze-drying microscopy to determine the critical transition temperature and therefore suitable freezing conditions for successful subsequent drying. Vacuum conditions should be selected based on the vapour pressure of ice at the chosen shelf temperature and the optimum temperature for early freeze drying at sub-ambient temperature (primary drying) should be selected based upon conditions that avoid the temperature of the reference material rising above that of the critical transition temperature determined for the formulation. In later stages (secondary drying), the temperature is ramped up to ambient temperature or higher to yield a reference material with low residual moisture. At the end of drying the standard should be stoppered in either a vacuum or a dry gas environment that will prevent the ingress of any atmospheric moisture into the container on storage. Antibody standards are typically stored under a dry, inert atmosphere such as nitrogen.

***Note***: the freeze-drying cycle for the First WHO International Standard for anti-SARS-CoV-2 immunoglobulin was as follows:

1. material was frozen at −50 °C for 4 hours
2. primary drying was carried out at −35 °C for 40 hours at 100 μbar vacuum
3. temperature was ramped up to 25 °C over 10 hours
4. secondary drying was carried out at 25 °C and 30 μbar vacuum
5. vials were back-filled with dry nitrogen at atmospheric pressure.

Examples of an SOP for filling an IS can be found in the published literature *(1, 9)*.

## 9. Characterization

Before a candidate secondary standard is calibrated against the IS, its identity should be confirmed using a suitable assay to demonstrate that it has the expected immunological activity. Examples of suitable assays include those used in the development of the First WHO International Standard for anti-SARS-CoV-2 immunoglobulin, as described in the WHO collaborative study report *(10)*.

Tests should also be performed on the candidate standard to evaluate the following attributes:

- Appearance – a freeze-dried standard should comprise a consistent, well-formed cake. Collapsed freeze-dried material is often associated with high residual moisture and poor stability. Any inconsistencies observed among individual containers should be investigated. The appearance of the reconstituted standard should be checked for consistency and the absence of particulate matter. Liquid and frozen standards should also be examined for their appearance and the absence of particulate matter.
- Moisture – low moisture content is critical for the long-term storage of freeze-dried standards. Ideally, for long-term storage stability, the moisture content of the standard should be < 1% – though higher levels of residual moisture may be acceptable for secondary standards provided that monitoring studies against a higher-order reference material indicate satisfactory stability.

- Potency – it should be demonstrated that the material in the container has retained its immunological activity for the assays in which it will be used. Where possible, the assays used should be based on WHO or compendial guidance (for example, European Pharmacopoeia or United States Pharmacopoeia). Other assays should be validated or qualified as appropriate.

Baseline parameters, such as moisture content and potency, should be set at this time to allow for evaluation and monitoring of the stability of the standard.

**Safety**

Antiserum standards should not pose a risk of infection to users or staff involved in their preparation. The bulk material should be shown to be free from bloodborne infectious agents using validated procedures, and this may be reaffirmed by testing material in the final container.

# 10. Calibration against the International Standard

## 10.1 Principles of calibration

Calibration is the process by which a concentration is assigned to a reference material (such as a secondary standard) by direct comparison against the measurements obtained using a higher-order reference, and represents a crucial stage in the establishment of a secondary standard. Each calibration of a candidate secondary standard should be performed in parallel with the higher-order reference (in this case, the WHO IS) using the same test. The following sections describe the minimum requirements for the calibration of secondary standards intended for use either by more than one laboratory using multiple methods (collaborative study calibration) or for a specific method in one laboratory (single laboratory calibration). In both cases, several independent runs with the candidate standard and the IS in parallel have to be performed (same assay using the same test conditions). For each run, a new vial of each standard should be used.

## 10.2 Collaborative study

The purpose of secondary antibody standards is to harmonize assays measuring defined analytes contained in the IS (for example, in terms of biological activity, antibodies binding different antigens or antigenic variants, or binding antibodies of different immunoglobulin types and specificities). Therefore, only assays measuring the same analyte that are validated (with regard to limit of blank, limit of quantitation, linearity, precision and analytical measuring interval) are included in the respective assessments. Assay harmonization and the commutability of the reference material are investigated through the inclusion of a set of various routine clinical samples (for example, representing different stages of infection, different infection courses, different antibody titres and antibody classes). However, in any given collaborative study, assays of different design and measuring different analytes may be included, provided that subsequent data analysis carefully differentiates between the individual analytes. A candidate secondary standard is considered to be fit for purpose only if both its capacity for harmonizing specific assays and its commutability are demonstrated by the collaborative study results.

Secondary antibody standards used by multiple laboratories (for example, different manufacturers and NCLs) should be calibrated directly against the current WHO IS in a

collaborative study. Ideally, the collaborative study should be organized in line with advice from a body with experience in this field, such as a WHO collaborating centre. If necessary, a scientific advisor from the field should be identified to support the collaborative study, including with regard to the selection of study participants. The calibration study should follow sound statistical principles (see section 11 below). Due to the complexity of the reported data, which typically include data from different types of assays, the statistical analysis should be performed by a statistician. The general principles of planning and conducting such collaborative studies are described in section A.6 of the WHO Recommendations for the preparation, characterization and establishment of international and other biological reference standards *(1)*.

The number of study participants will depend on the nature of the study, its aims, the number and type of assay systems to be used, the materials to be studied, and the availability and resources of suitably experienced participants. For a secondary standard, both the number and geographical spread of participants are likely to be more limited than for a global collaborative study to establish an IS. The laboratories participating in the collaborative study will need to have experience in some or all of the assays in which the secondary standard will be used. For some standards, this may restrict the number of potential participants but, in principle, there should be sufficient participants to generate an adequate number of datasets when assays are variable. Where there are few participants, a larger number of independent assay runs may be required to ensure sufficient precision of the assigned potency. Ideally, in addition to the various assays performed in the participating laboratories, there should be an assay performed by all participants, with the SOP provided in advance by the WHO collaborating centre. Alternatively, the compliance of the participating laboratories with the relevant ISO standard may suffice.

Prior to the start of the study, acceptance criteria regarding precision, linearity, limit of quantitation and analytical measuring interval should be obtained, along with respective information on the assays that participants are proposing to use. In addition to the study invitation and response forms, a study protocol should also be sent to all participants outlining the results and other information to be returned. Appendix 3 provides model document templates for all of the above purposes, along with a proposed outline template for the final collaborative study report.

### 10.3 Single laboratory calibration

In some cases, the calibration may be carried out by a single laboratory with experience of the relevant assay(s) – for example, a vaccine manufacturer, assay manufacturer or local NCL. The assay used should be analytically validated (with regard to limit of blank, limit of quantitation, linearity, precision and analytical measuring interval). In such circumstances, a larger number of independent assay runs may have to be performed to ensure acceptable uncertainty with respect to the assigned potency (see sections 11.2 and 11.4 below).

## 11. Statistical analysis

The following sections are based on a common statistical method used for the calibration of reference materials – that is, parallel-line (or curve) analysis as described by WHO *(11)* and the European *(12)* and United States *(13)* pharmacopoeias. Another common method is based on demonstrating linearity of the primary and secondary reference materials in the assay system used *(8)*. Any statistical method that has been demonstrated to be a reliable approach to the calibration of such materials can be applied. Appropriate software for the statistical analysis should be available for the evaluation of the data, and the statistical analysis should

be performed by staff with expertise in this field. Examples of software used for such statistical analyses are provided in Appendix 4.

## 11.1 Statistical models

The calibration study data should be analysed using the relevant statistical model for the assay. The statistical validity of the fitted model should be assessed for each individual assay run. For the parallel-line and probit models, the linearity and parallelism of the logarithmic dose–response relationships between the IS and secondary standard should be evaluated and shown to meet the system and sample suitability criteria before the potency of the candidate secondary standard relative to the IS can be calculated. Parallelism could be demonstrated by means of a significance test for non-parallelism *(12)* – though an equivalence approach for the difference or ratio of slopes may be preferred (that is, the confidence interval for the ratio of slopes must entirely lie between predefined equivalence margins). In addition, the precision with which potency has been estimated should be provided, usually in the form of a 95% confidence interval for the estimate.

Each calibration will have a stated measurement uncertainty (see section 11.4 below). This estimate can be determined by identifying all sources of variation, calculating the extent of variation and using established methods to combine the uncertainty. The measurement uncertainty associated with assigning a value to the standard is test-system specific. It should be noted that an IS, by definition, has a specified value which has typically been assigned and expressed in IU/mL. As a consequence of defining the IU as a fraction of the contents of the container of the current IS, and because the units defined by any previous IS formally cease to exist, an uncertainty value is not given to the assigned IU *(1)*. The variability of the vial weight during filling for each IS is stated in the study report and given in the Instructions for Use (IFU) accompanying the standard.

## 11.2 Collaborative study calibration using multiple assays

Results from all participants should be analysed using statistical methods described and considered appropriate by the responsible statistician. This analysis typically requires access to suitable computing facilities and statistical software (see Appendix 4). The testing requirements and protocol of each laboratory/test should follow the protocol described for the single laboratory calibration (see section 11.3 below). The results of each assay method should be analysed separately and should provide an estimate of the relative potency, with associated uncertainty, of the candidate secondary standard against the IS.

The variations observed in the results for different test methods, and across different laboratories, should be described and assessed as part of the statistical analysis (to determine the precision and consistency of the results). An assessment should be made of any factors causing significant heterogeneity of the estimated potency, non-linearity or any differences in slopes. Although there is no generic outlier-detection rule from a statistical point of view, the exclusion of data should be taken into account in subsequent analysis wherever striking differences are observed in results within assays, between assays, between participants or between test methods. All valid potency estimates for the candidate secondary standard should be combined to produce an arithmetic mean or geometric mean potency with 95% confidence limits. It is useful to display and assess the results graphically – for example, as histograms or scatter plots.

## 11.3 Single laboratory calibration

The IS and candidate secondary standard should be tested in a minimum of three independent assay runs. The candidate material should be tested neat (where possible) and at a minimum of two further (for example, twofold) dilutions within the linear range of the assay. The same methodology applies to the IS with the exception that this material should be diluted starting from a concentration as close as possible to the estimated potency of the secondary standard (as indicated by preliminary tests). All standards should be tested at a minimum in duplicate, taking into consideration the precision of the assay. The results obtained from the parallel-line analysis should be used to give the "relative potency" of the secondary standard against the IS in IU/mL. Parallel-line or curve analysis should be the preferred option for data analysis.

### 11.4 Calculation of measurement uncertainty

The assignment of an uncertainty value must be considered for the calibrated value applied to secondary reference materials. The uncertainty of an observed value is a property of the test system and is not the effect of mistakes introduced through human error. The calculation of uncertainty is a complex area and advice should be sought from a statistician.

Such uncertainty – often referred to as "measurement uncertainty" (MU) – expresses the 95% confidence limits either side of the observed value assigned to a material. Estimating the MU of a reference standard indicates the degree of confidence in the value assigned. Where no MU is assigned, justification for this should be provided (for example, when the calculated uncertainty is negligible in comparison to the known variability of the assay in which the standard will be used).

There are many aspects to uncertainty and well-documented examples of how to estimate it *(14, 15)*. One typical approach to estimating MU for a secondary standard is to test the material multiple times on different occasions (but always using the same test system) in parallel with the WHO IS (that is, under the exact same conditions) and then combine the results from at least three independent test runs. The more times the sample is tested the better it will be in terms of reducing the magnitude of MU. For the calibration of a single assay, the test system used should be of the highest possible quality – that is, a commercial assay or, in the absence of such, a well-validated laboratory-developed test. Estimated MU (95% confidence limits) for potency estimates can be calculated using the usual statistical methods *(14)* which account for the observed intra-assay and inter-assay variation. This approach demonstrates the imprecision but does not account for MU derived from inherent bias.

When developing and assigning a value to any secondary reference standard, evaluating the likely impact of accumulated uncertainty, and determining whether this is acceptable given the intended use of the standard, will be an important element in the study design. For example, where a secondary standard is intended to calibrate an assay that has a high degree of precision, or is used to make a medical decision within a very narrow range of results, then a high degree of uncertainty regarding the value assigned to the secondary standard may not be appropriate. In such cases, careful selection of suitable measurement procedures may be required to minimize the uncertainty associated with the value assigned to the secondary standard. This is particularly important where the secondary standard will be used to assign a value to a manufacturer's internal standard since this will lead to the further accumulation of uncertainty.

## 12. Stability

Understanding the stability of a reference standard is important for: (a) estimating its shelf-life in storage for its intended use; (b) identifying appropriate conditions for distribution to users; and (c) determining its shelf-life following reconstitution. Evidence of continued stability can be acquired from the reports and feedback of users and by monitoring long-term stability, in real time, against the assigned potency of the IS. The application of predictive models of stability (such as the Arrhenius model), which are used during the development of an IS where there is no higher-order reference material, is not generally necessary with secondary standards, the stability of which can be assessed by reference to the IS. In general, the antibody activity of freeze-dried antisera and plasma is stable at −20 °C for a specific time period, which should be included in the IFU of the secondary standard material under "recommended storage conditions". Where this is not the case, the stability of frozen or liquid preparations should be determined experimentally in real time by the manufacturer of the secondary standard material prior to making the material available to users.

Reference standards should be granted official status for use on the basis of the available data, including data on the long-term stability of the material, the consistency of the data generated in the assay and the outcomes of regular assessments performed against the IS. The date of preparation of the material should be indicated on each container and a batch validity statement should be available for each reference standard.

## 13. Monitoring stability in storage

Secondary antibody standards should be stored at an appropriate temperature, established by the stability studies conducted during its development. The temperature of the storage facility should be monitored and recorded routinely (for example, by using an automated temperature monitoring system), and alternative storage arrangements should be available in case of breakdown.

***Note***: The use of frost-free freezers is not recommended as the temperature cycles vary more widely than those of freezers that are defrosted manually.

A protocol for monitoring the stability of the standard during storage should be developed. This may include obtaining data generated during use of the standard from as many users as possible (for example, data on neutralizing antibody or antibody binding titres). However, such "supplementary" information should not be used to establish the adequate storage conditions of the standard material. Where the data indicate a possible stability issue, further investigations should be undertaken by the custodian laboratory – such as a small collaborative study involving laboratories familiar with the use of the standard.

The stability of the standard should be assessed periodically relative to the IS or to the baseline if the IS is not available. The frequency of assessment required will be dependent on the monitoring data and the predicted stability of the standard.

## 14. Responsibilities of the custodian laboratory

Once the secondary standard has been established, the custodian laboratory is responsible for the following:

- Storage of the secondary standard under appropriate conditions established during development.
- Distribution of the secondary standard when requested under appropriate conditions established during development.

- Maintenance of complete records on the project, covering:
  - the source of the bulk standard and its characterization, before and after filling;
  - collaborative study protocol, results, statistical analysis and report;
  - results of stability studies;
  - storage, inventory and dispatch of the reference standard;
  - number of ampoules/vials of standard established and distributed;
  - recipients of the standard in case any issues arise that would require all users to be informed.
- Documentation of feedback from users.
- Maintaining awareness of relevant assay developments and of how the standard is being used.
- Monitoring stability by requesting feedback on the use of the secondary standard that might provide ongoing evidence of the stability of the material.
- Publishing the results of the collaborative study.
- Providing advice and training on the use of the standard.

The custodian laboratory may consider implementing an MTA to ensure the appropriate use of the standard by the recipient and to address any safety issues associated with its shipment, storage and use.

## 15. Instructions for use and labelling

All ampoules or vials of the secondary standard should be labelled with the name of the custodian laboratory, the name of the material, any assigned code number, the assigned potency, the storage temperature and a clear indication that the material is ”Not for use in humans”. If an expiry date is assigned, this must also be clearly stated on the label.

Each package of secondary standard should include a data sheet/IFU containing the following information:

- the storage and shipping conditions;
- the potency of the secondary standard;
- the assays in which it may be used;
- instructions on the reconstitution of the secondary standard;
- a statement confirming the stability of the secondary standard under appropriate conditions of transport;
- relevant safety information;
- clearly specified information on stability (which should be updated should further evidence become available);
- date of production;
- if frozen liquid, the volume should be stated;
- if an expiry date is assigned, this must be clearly stated;
- stability of the secondary standard once reconstituted, diluted or aliquoted;
- contact information for feedback on any issues relating to the use, quality or stability of the secondary standard; and
- reference to the collaborative study report.

If the estimate of the potency and MU of the secondary standard relative to the IS is assay and/or antigen specific, this should be clearly stated in the IFU as it will affect the use of the secondary standard.

Once the secondary standard has been reconstituted, diluted or aliquoted, users should be advised to determine the stability of the material according to their own methods of preparation, storage and use. A standard cannot be stored indefinitely at 4 °C. Therefore, aliquots prepared aseptically should be frozen until used and not freeze-thawed, and once an aliquot is opened it should be kept at 4 °C.

## 16. Dispatch of standards

Standards should be dispatched under conditions appropriate to the stability of the standard so that its potency is not affected during shipping. The anticipated time in transit and at ambient temperature should be considered. Standards that are stored frozen should be dispatched on dry ice overnight to avoid multiple freeze-thaw cycles unless stability studies have shown this to be unnecessary. The IFU should also contain a separate statement confirming the stability of the reference standard under the conditions of transport.

Standards should be packaged and dispatched according to international regulations and import permits relating to the safety of biological materials. This should take into account any residual risk that the material is infectious and be documented for each standard.

## 17. Secondary standard replacement

The replacement of a secondary standard needs to be planned and to be timely. The processes described in this manual should be followed, including calibration of the replacement material against the IS and not the previous secondary standard. Nevertheless, even when an IS is used for calibration, the inclusion of the previous standard in the study can still provide a useful indicator of assay performance. Although the previous secondary standard may thus be included in the study, it should not be used for the calibration of the replacement material as this will increase the risk of the assigned value drifting. Only in cases where an IS is not available should the calibration be made against the previous batch of secondary standard.

If surplus plasma or pooled sera are available from the original study (and have been stored under appropriate monitored conditions) this material could be used to allow for replacement with an identical material.

The approach to be taken to the replacement of a secondary standard should be planned and described as part of the initial proposal for the original establishment of the material.

## 18. Authors and acknowledgments

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## 19. References


1. Recommendations for the preparation, characterization and establishment of international and other biological reference standards (revised 2004). In: WHO Expert Committee on Biological Standardization: fifty-fifth report. Geneva: World Health Organization; 2005: Annex 2 (WHO Technical Report Series, No. 932; https://www.who.int/publications/i/item/9241209321, accessed 30 March 2022).

2. WHO manual for the establishment of national and other secondary standards for vaccines. Geneva: World Health Organization; 2011 (Document WHO/IVB/11.03; https://www.who.int/publications/i/item/WHO-IVB-11.03, accessed 30 March 2022).

3. WHO manual for the preparation of secondary reference materials for in vitro diagnostic assays designed for infectious disease nucleic acid or antigen detection: calibration to WHO International Standards. In: WHO Expert Committee on Biological Standardization: sixty-seventh report. Geneva: World Health Organization; 2017; Annex 6 (WHO Technical Report Series, No. 1004; https://www.who.int/publications/i/item/9789241210133; accessed 30 March 2022).

4. First WHO International Standard for anti-SARS-CoV-2 immunoglobulin; and First WHO International Reference Panel for anti-SARS-CoV-2 immunoglobulin. In: WHO Expert Committee on Biological Standardization: report of the seventy-second and seventy-third meetings. Geneva: World Health Organization; 2021 (WHO Technical Report Series, No. 1030, section 9.1.2, pp. 66–8; https://www.who.int/publications/i/item/9789240024373, accessed 30 March 2022).

5. Bewley KR, Coombes NS, Gagnon L, McInroy L, Baker N, Shaik I et al. Quantification of SARS-CoV-2 neutralizing antibody by wild-type plaque reduction neutralization, microneutralization and pseudotyped virus neutralization assays. Nat Protoc; 2021;16:3114–40 (https://www.nature.com/articles/s41596-021-00536-y, accessed 30 March 2022).

6. Nie J, Li Q, Wu J, Zhao C, Hao H, Liu H et al. Quantification of SARS-CoV-2 neutralizing antibody by a pseudotyped virus-based assay. Nat Protoc. 2020;15:3699–715 (https://www.nature.com/articles/s41596-020-0394-5, accessed 30 March 2022).

7. International vocabulary of metrology – basic and general concepts and associated terms (VIM). Third edition. 2008 version with minor corrections. Joint Committee for Guides in Metrology; 2012 (Document JCGM 200:2012 (E/F); https://www.bipm.org/documents/20126/2071204/JCGM_200_2012.pdf, accessed 30 March 2022).

8. Vesper H, Emons H, Gnezda M, Jain CP, Miller WG, Rej R et al. Characterization and qualification of commutable reference materials for laboratory medicine – approved Guideline. Wayne (PA): Clinical and Laboratory Standards Institute; 2010 (Document EP30-A; available from: https://clsi.org/standards/products/method-evaluation/documents/ep30/, accessed 30 March 2022).

9. Matejtschuk P, Malik K, Duru C. Formulation and process development for lyophilized biological reference materials. In: Ward KR, Matejtschuk P, editors. Lyophilization of pharmaceuticals and biologicals. Part of the Methods in Pharmacology and Toxicology book series. New York (NY): Humana Press; 2019: pp. 33–55 (available from: https://link.springer.com/protocol/10.1007/978-1-4939-8928-7_2, accessed 30 March 2022).

10. Establishment of the WHO International Standard and Reference Panel for anti-SARS-CoV-2 antibody. Geneva: World Health Organization; 2020 (WHO/BS/2020.2043; https://www.who.int/publications/m/item/WHO-BS-2020.2403?msclkid=dc090727c85611eca0a610ca076ad3e6, accessed 30 April 2022).

11. Manual for quality control of diphtheria, tetanus and pertussis vaccines. Geneva: World Health Organization; 2013 (WHO/IVB/11.11; https://apps.who.int/iris/rest/bitstreams/284404/retrieve, accessed 9 May 2022).

12. Statistical analysis of results of biological assays and tests. 01/2008:50300. European Pharmacopoeia 6.0, chapter 5.3 (http://www.uspbpep.com/ep60/5.3.%20%20statistical%20analysis%20of%20results%20of%20biological%20assays%20and%20tests%2050300e.pdf, accessed 30 March 2022).

13. Analysis of biological assays. General Chapter (1034). Rockville (MD): United States Pharmacopeia; 2012 (Introduction: https://doi.usp.org/USPNF/USPNF_M5677_01_01.html?msclkid=5f037abecfa911ec84ff300e282f58cc, accessed 9 May 2022).

14. Bell S. A beginner's guide to uncertainty of measurement. Measurement good practice guide No. 11 (Issue 2 with amendments March 2001). Teddington: National Physical Laboratory; 1999 (https://www.npl.co.uk/special-pages/guides/gpg11_uncertainty, accessed 30 March 2022).

15. Dimech W, Francis B, Kox J, Roberts G. Calculating uncertainty of measurement for serology assays by use of precision and bias. Clin Chem. 2006;52(3):526–9 (https://academic.oup.com/clinchem/article/52/3/526/5626796?login=false, accessed 30 March 2022).

# Appendices

The following appendices have been provided by institutions with extensive experience of producing biological measurement standards, and present examples of potentially useful processes and procedures for laboratories developing, storing and distributing secondary measurement standards for antibodies. They are not intended to be prescriptive and may be adapted by such laboratories as required.

Appendices 5–10 provide specific standard operating procedures (SOPs) for established bioassays that are known to work at their respective donor institution, and with appropriate adaptation to take account of local facilities and procedures, may be used by other laboratories developing secondary antibody standards. Although these SOPs are largely as provided to WHO, references to highly specific local details, resources and regulations have been removed.

# Appendix 1

## Preparation and calibration of national standard substances of biologics

### I. Definition

National standard substances of biologics refer to the biological standards or references used to determine the potency, toxicity or content of biological products to identify and characterize them.

### II. Classification of national standard substances

National standard substances are divided into two classes:

1. **National Biological Standards** refer to the standard substances calibrated with international standards or prepared domestically (if international standards are not available) which can be used to measure the potency, toxicity or content of a given product. Content is expressed in SI units – for example, milligram (mg). Biological activity/potency is expressed in international units (IU), specific activity units or units.
2. **National Biological References** refer to biological diagnostic reagents, biomaterials or specific antisera calibrated with international reference reagents or prepared domestically (if international reference reagents are not available) which can be used for the qualitative identification of microorganisms (or their derivatives) or as disease reference materials for the quantitative determination of biological activity/potency of certain biological products, for example, reference materials used for titration of virus content in live measles vaccine, or of flocculation units of toxoid, by which the activity/potency can be expressed in specific activity units or in units rather than in IU.

### III. Preparation and calibration of national standard substances

1. Laboratories and clean rooms used to prepare national standard substances of biologics shall comply with the requirements of good manufacturing practices (GMP) for pharmaceutical products and good laboratory practices (GLP).
2. The National Control Laboratory (NCL) is responsible for calibrating national biological standard substances.
3. Research and development of new national standard substances:
   - **Selection of source materials** – the nature of source materials for national standard substances shall be identical to that of the sample to be tested. Source materials shall not contain any interfering contaminants. Source materials shall be sufficient in quantity and of adequate stability and high specificity.
   - **Filling containers** – filling containers shall be neutral borosilicate glass. Heat sealing of the ampoule after the freeze-dried standard substance has been added will improve the stability of the standard substance.
   - **Formulation, filling, lyophilization and sealing of containers** – the formulation and dilution of standard substances shall be performed as required. Any necessary stabilizers or other materials shall not affect the activity, stability or assaying processes of the standard substance, and shall not volatilize during lyophilization. Substances qualified in control tests shall be

dispensed accurately with a precision of ± 1%. Substances that need to be dried for preservation shall be sealed immediately after lyophilization. Residual moisture in the freeze-dried substances shall not exceed 3.0%. It is necessary to ensure consistency in terms of the potency and stability of the substance in each container during the course of filling, lyophilization and sealing.

- **Test items** – test items shall be appropriate to the characteristics and intended purposes of the standard substances used, including at least, but not limited to, tests for filling precision, residual moisture, sterility, biological activity/potency and stability.
- **Calibration** – ***collaborative calibration***: the development and calibration of standard substances to be established shall be conducted collaboratively in at least three experienced laboratories. The participants shall adopt the same protocols, and statistical analysis of the calibrated results shall be performed (the calibrated results necessitate at least five independent valid results). ***Confirmation of activity*** (potency unit or toxicity unit): activity is typically expressed as the mean value of the calibrated results obtained by participating laboratories. Data from the collaborative calibration shall be collected and analyzed statistically by the NCL. Standard substances shall be assigned their activity value using appropriate statistical analysis methods and officially released following approval.
- **Stability studies** – accelerated stability tests shall be performed during the development step. Candidate substances shall be placed at various temperatures (−20 °C, 4 °C, 25 °C and 37 °C) for further testing of biological activity or content. The activity or content of established standard substances shall also be checked periodically.

4. Preparation and calibration of a substitute lot of standard substance:
   - The NCL shall be responsible for preparation and calibration.
   - The biological properties of the source materials used to prepare the substitute lot of standard substance shall be as similar as possible to those of the substituted lot.

## IV. Approval of standard substances

1. The collaboratively calibrated results for a newly established standard substance shall be reviewed by the NCL.
2. Substitute lots of standard substances shall be reviewed by the NCL.
3. The newly established standard substance (or substitute lot) shall be released for use only after obtaining approval from the Standards Review Committee.

## V. Labels and package inserts

1. Labels and package inserts shall be issued for qualified standard substances by the quality assurance department of the NCL.
2. The label shall indicate the name, code number, lot number, extractable volume, usage, storage conditions, manufacturer name, etc.
3. Package inserts shall be provided for each standard substance or reference material, and shall include the information given on the label, along with information on the components and characteristics of the substance/material, usage method, stability, etc. If necessary, any relevant references shall also be provided.

## VI. Use, release and storage of standard substances

1. National standard substances of biologics shall apply in the implementing of national standards for drugs. The evaluation of a national standard substance of biologics shall be valid only for its specified usage. If used for other purpose, its applicability shall be confirmed by the user themselves.
2. Requests for national standard substances of biologics should be made directly to the NCL. National standard substances are provided to manufacturers to calibrate working standards or for quality control purposes.
3. National standard substances of biologics shall be stored at an appropriate temperature and humidity, which shall be periodically monitored and recorded.
4. A designated person shall be responsible for managing and releasing national standard substances of biologics.

# Appendix 2

## Documentation to be compiled during a standardization project

- Information on plasma pool – for example, source of individual donations, characterization of donations, ethical approval and other relevant correspondence.
- Characterization technical records (fill logs/details).
- Collaborative study raw data.
- Collaborative study reports.
- Documentation recording the decision-to-establish by appropriate authority.
- In-use scientific feedback (including on stability).

# Appendix 3

## Collaborative study documentation

Model templates for Invitation, Response form and Collaborative study protocol

### I: Model template: Invitation

Dear……..

I am writing on behalf of [.....] to invite you to participate in a collaborative study to establish a national/regional measurement standard for [.....]. The aims, provisional structure and timelines of the study are set out in the attached study protocol. The study will involve testing the International Standard (IS) and [*n*] candidate antibody standards in [*n*] assays.

We would ask you to:

1. Confirm if you are able to participate in this study.
2. If so, please complete the attached questionnaire.
3. Provide comments on the proposed study protocol relevant to your contribution.

Please note, it is normal practice to acknowledge study participants as contributors of data rather than co-authors in publications describing the establishment of the standard. Individual participants' data will be coded and reported "blind" to other participants during the preparation of the study report, and in subsequent publications.

Thank you for considering this request. We hope you are able to participate.

Yours sincerely,

[.....]

**II. Model template: Response form**

Name of participant:

Address:

Telephone number:

Email:

I would like to/am unable to* participate in the collaborative study to assess the suitability of the candidate reference material to serve as a secondary standard/national standard for [.....].

* Delete as appropriate.

If able to participate, please provide:

- any additional information required for shipping materials
- brief description of method to be used
- antibody assays routinely performed.

Signed:

Name:

Date:

Please return to: [.....]

Email: [.....]

## III: Model template: Collaborative study protocol

### Background

Including need for the standard, availability of IS, information on IS, and specifications of the candidate material

### Information on materials to be included in the study

International Standard
Candidate secondary standard
Any other samples

Include any advice on storage, biosafety, reconstitution (if freeze-dried) etc.

### Assay methods

WHO/pharmacopoeial and/or methods in use in laboratory

### Design of study

Number of assays
If more than two samples (IS + candidate secondary standard) are being tested, emphasize need to include all study samples in each assay
Indicate appropriate dilutions for the study samples

### Results and data analysis

Supply data sheet so that all essential information can be recorded
A separate data sheet should be completed for each test run

### Timelines

Include deadlines for the return of results

### Result sheet – showing title of study

Participant
Laboratory
Date of assay
Method – WHO, in-house, other
Participant's calculation of potency of each serum sample in IU
For each serum sample – dilutions tested, method used, responses (OD, plaque number etc.), and data from relevant controls

Please return to: [.....]

Email: [.....]

## Collaborative study report – outline of required contents

### Introduction

Including background, the need for the secondary standard and the study aims.

### Materials

- Candidate secondary standard – description of source of plasma, including ethical considerations; whether individual donors were convalescent or vaccinated; how donations were treated; characterization of individual donations; how donations were pooled and the rationale for excluding particular donations; any treatments applied to the bulk pool (such as defibrination); the identifying code of the candidate secondary standard.
- Other study samples.
- Name and code of the IS against which the candidate secondary standard was calibrated.

### Participants

List participants and their locations.

### Study design and assay methods

- Set out study design and refer to the study protocol
- Indicate which assay(s) were used in the study
- Indicate validation requirements
- Indicate the analytical measuring interval or linear range of assay(s)
- Indicate suggested dilutions for materials
- Provide plate template
- Include the number of assays that participants were requested to perform
- Describe the stability study.

### Results

Include statistical and other analysis, identity-blinded if appropriate, of:

- the numbers of valid and invalid results;
- the grounds for any exclusion of outlier results (for example, non-parallelism or nonlinearity);
- comparison of assay results for materials tested by different assay methods, together with their interpretation and comments on particular factors (such as the frequency distribution of the estimates, differences in potency estimates and any observed factors which may account for these), and differences observed between different assay methods;
- the within-assay variation for each laboratory using a given assay method and the overall between-assay variation where possible;
- the overall estimates of relative potencies for each assay method, calculated both with and without outlying results;
- the final figure for the overall estimate of the potency of the proposed secondary standard, comments on the validity of this estimate, and if appropriate, the 95% confidence intervals and the method of deriving them; and
- stability data.

**Discussion/conclusions**

**Proposed value assignment**

**Tables and figures**

# Appendix 4

## Software for statistical analysis of bioassay data

There are many commercial software packages that are suitable for the evaluation of data and statistical analyses generated by calibration studies. The choice of which software to use should be made in consultation with staff with expertise in this field. The following are examples of publicly available packages widely used in the calibration of biological standards.

### WHO Bioassay Assist

Bioassay Assist is a statistical analysis software package used for the quality control of biological products donated to WHO by the National Institute of Infectious Diseases, Japan, and is available to users agreed by WHO. The software provides both calculation and data-analysis functions – including parallel line and probit assays, the two methods most frequently used in bioassays.

Subject to WHO approval, the software is provided free upon request from:

Dr Dianliang Lei – leid@who.int
Norms and Standards for Biologicals
Technical Standards and Specifications Unit
Health Products Policy and Standards Department
Access to Medicines and Health Products Division
World Health Organization, Avenue Appia 20, CH-1211 Geneva 27, Switzerland.

### CombiStats™

This software package is intended for use in the statistical analysis of data from biological dilution assays or potency assays. It includes parallel line, slope ratio, probit, 4- and 5-parameter logistic curve, and single-dose models, as well as $ED_{50}$ calculations.

The software is available from the European Directorate for the Quality of Medicines & Healthcare (EDQM), Council of Europe, upon payment of an annual licence fee.

For further information please see: www.edqm.eu/en/combistats

### Other useful software includes:

- ELISA for Windows – available from the Centers for Disease Control and Prevention, Atlanta, GA, the USA at: https://www.cdc.gov/ncird/software/elisa/index.html
- The IU ELISA Calculator provided by the Karolinska Institute, Sweden at: http://188.114.242.3:8080/IUWeb/

# Appendix 5

## SOP of ELISA for SARS-CoV-2 antibodies[1]

### Summary

An in vitro enzyme-linked immunosorbent assay (ELISA) is used to assay the binding of human antibodies/sera to recombinant antigens. This ELISA is a non-competitive direct binding assay. First, antigen is coated onto a microplate and then plasma/sera samples are added. Any bound antibody from these samples then binds to an appropriate anti-species peroxidase-conjugated antibody. The antibody complex is then detected with a TMB substrate.

### Health and safety

Follow local health and safety regulations; wear suitable personal protective equipment as stipulated in the relevant risk assessment (for example, laboratory coat, nitrile gloves and eye protection).

### Equipment and materials

- Flat-bottom NUNC maxisorp 96-Well Plates (Fisher Scientific cat. no. 44-2404-21, or equivalent);
- Phosphate buffered saline (1X) (Gibco cat. no. 10010-023, or equivalent);
- Tween 20 (Fisher Bioreagents cat. no. BP337-500, or equivalent);
- Milk powder (Marvel, or equivalent);
- TMB substrate (Neogen cat. no. 309175, or equivalent);
- 1N sulphuric acid $H_2SO_4$ (Sigma-Aldrich cat. no. 339741, or equivalent);
- Polypropylene sterile conical tubes: 15 mL and 50 mL;
- Sterile, serological pipettes: 5 mL, 10 mL and 25 mL;
- Micropipette tips: 10 µL, 20 µL, 200 µL, 500 µL and 1000 µL;
- Sterile reservoirs (Fisher Scientific cat. no. 07-200-127, or equivalent);
- Multichannel pipette(s): 200 µL;
- Wypalls;
- Pipetboy (Integra Biosciences), or equivalent;
- Class II biological safety cabinet;
- Ultra-low freezer (−80 °C);
- Refrigerator at 4 °C (± 1 °C);
- Wellwash™ Versa Microplate Washer (Thermo Scientific) or equivalent; and
- FLUOstar® Omega Microplate Reader (BMG Labtech) or equivalent.

**Proteins**

This protocol was set up using the following proteins:

- NIBSC/CFAR (kindly donated by Dr P. Cherepanov, The Francis Crick Institute, London, the United Kingdom) based on the original SARS-CoV-2 isolate MN908947 (https://www.ncbi.nlm.nih.gov/nuccore/MN908947);

[1] Example of an SOP for an established bioassay that may be adapted for use by laboratories developing secondary antibody standards.

- SARS-CoV-2 S1 (#100979);
- SARS-CoV-2 RBD (#100981);
- SARS-CoV-2 N (#100982); and
- SARS-CoV-2 trimeric spike (#101007) produced by C. Ball (NIBSC) using plasmid obtained from Dr B. Graham (NIH/NIAID, Bethesda, MD, the USA).

Other source for plasmids or proteins, including for variants of concern: BEI Resources: BEI Highlights
(https://www.beiresources.org/BEIHighlights1.aspx?ItemId=79&ModuleId=14004)

**Antibodies**

- Secondary antibody: anti-human IgG (Fab specific)-peroxidase antibody produced in goat (Sigma cat. no. A0293) (use at 1 in 3000);
- Positive control for anti-S1/RBD/Spike: anti-COVID-19 and SARS-CoV S glycoprotein; [CR3022], human IgG1, Kappa (Absolute antibody, Ab1680.10) positive control – dilute to 0.5 µg/mL;
- Positive control for nucleoprotein: SARS-CoV-2 (2019-nCoV) Nucleoprotein/NP; antibody, rabbit mAb (Sino Biological, 1018140143-R019-SIB) – dilute to 0.5 µg/mL; and
- Related secondary antibody: anti-rabbit HRP (Sino Biological, G33-62G-SGC) – use at 1 in 10 000.

## Procedure

### *Day 1: Coating ELISA plates*

1. Coat NUNC maxisorp ELISA plate with 50 µL of antigen at 1 µg/mL diluted in 1X PBS.
2. Gently tap the plate to make sure that the wells are covered.
3. Incubate overnight at 4 °C, covered.

All following steps to be carried out at room temperature (21 °C ± 3 °C).

### *Day 2: ELISA assay*

1. Wash plate 3 times with PBS/0.05% Tween 20 (v/v).
2. Block with 200 µl of PBS/0.05% Tween 20 (v/v) with 5% milk.
3. Incubate at room temperature for 1 hour, covered.
4. Prepare serum samples to 1:100 diluted in PBS/0.05% Tween 20 (v/v) with 5% milk.
5. Wash plate 3 times with PBS/0.05% Tween 20 (v/v) (wash buffer).
6. Add 50 µL PBS/0.05% Tween 20 (v/v) with 5% milk to all wells in rows B–H, columns 2–11.
7. Add 75 µL of each diluted sample to the relevant wells in row A, columns 2–11.
8. Add 50 µL of positive and negative controls diluted appropriately in PBS/0.05% Tween 20 (v/v) with 5% milk to the relevant wells in columns 1 and 12.
9. Using a multichannel pipette, titrate samples threefold down the plates by removing 25 µL from row A and transferring into row B and mixing. Repeat this stepwise down the plate (row B to C, C to D etc.); discard 25 µL from final row.
10. Incubate at room temperature for 1 hour, covered.

**NB plate layout shows samples tested in duplicate**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | Dilution |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | Positive control | Sample1 | Sample1 | Sample2 | Sample2 | Sample3 | Sample3 | Sample4 | Sample4 | Sample5 | Sample5 | Blank | 1:100 |
| **B** | Positive control | | | | | | | | | | | Blank | 1:300 |
| **C** | Negative control | | | | | | | | | | | Blank | 1:900 |
| **D** | Negative control | | | | | | | | | | | Blank | 1:2700 |
| **E** | Blank | | | | | | | | | | | Negative control | 1:8100 |
| **F** | Blank | | | | | | | | | | | Negative control | 1:24 300 |
| **G** | Blank | | | | | | | | | | | Positive control | 1:72 900 |
| **H** | Blank | | | | | | | | | | | Positive control | 1:218 700 |

11. Wash plate 3 times with PBS/0.05% Tween 20 (v/v).
12. Add 50 µL of anti-human IgG (Fab specific) horseradish peroxidase-conjugated secondary antibody diluted 1:3000 in PBS/0.05% Tween 20 (v/v) with 5% milk.
13. Incubate at room temperature for 1 hour, covered.
14. Wash plate 3 times with PBS/0.05% Tween 20 (v/v).
15. Add 50 µL TMB to all wells.
16. Allow to develop for 10 minutes.
17. Stop the reaction after 10 minutes by adding 50 µL of 2M $H_2SO_4$ to all wells.
18. Read at 450 nm absorbance on a plate reader immediately.

***Note***: this assay could be adapted for S1, RBD or spike protein IgM and IgA determination also, using the following antibodies as controls and secondaries:

**IgM**

- anti-COVID-19 and SARS-CoV S glycoprotein [CR3022], human IgM, Kappa (Absolute antibody, Ab1680.15) dilute to 0.5 µg/mL; and
- anti-human IgM (µ-chain specific) peroxidase antibody produced in goat (Sigma cat. no. A0420) (use at 1 in 3000).

**IgA**

- anti-COVID-19 and SARS-CoV S glycoprotein [CR3022], human IgA, Kappa (Absolute antibody, Ab1680.16) dilute to 0.5 µg/mL; and
- anti-human IgA (α-chain specific) peroxidase antibody produced in goat (Sigma cat. no. A0295) (use at 1 in 3000).

# Appendix 6

## Microneutralization assay for coronaviruses[2]

### Purpose

This SOP describes a method for quantifying the neutralizing activity of antibodies against coronaviruses such as SARS-CoV-2 and MERS-CoV. Following incubation of the virus with serial dilutions of serological material and addition to a permissive cell line, the level of infectivity is read 2 days later by staining cells for expression of the coronavirus spike or nucleoprotein. The readout is measured in optical density (OD) units.

All local health and safety regulations for handling coronaviruses should be followed. In the United Kingdom (as of January 2022) SARS-CoV, SARS-CoV-2 and MERS-CoV are classified as hazard group 3 by the Health and Safety Executive (HSE) Advisory Committee on Dangerous Pathogens.

Appropriate risk assessments, standard operating procedures and other relevant documents such as a Biological Safety Data Sheet should be in place ahead of commencing any work. All work with a live virus must be carried out inside a microbiological safety cabinet (MSC).

### Materials

- Gilson p20, p200, p1000 pipettes (or equivalent)
- Multichannel pipettes 20–200µl (or equivalent)
- Pipette tips
- Wypalls
- Sealable secondary containers (for example, sandwich box)
- Tissue culture treated flat bottom (FB) 96-well plates (ThermoFisher, cat. no. 10334791)
- Sterile U bottom 96-well plates (ThermoFisher, cat. no. 10520832).

### Reagents

- Appropriate disinfectant (for example, Microsol4 10% in water, Anachem, cat. no. 30312915); and
- Industrial methylated spirit (IMS) 70% (v/v) in water or equivalent alcohol-based disinfectant.

Reagents and cell culture media to be used in the following procedures are cell type and cell line specific – examples of the most commonly used media are given below.

- Growth medium – Dulbecco's MEM (Sigma, cat. no. D6546) or equivalent, supplemented with **10% fetal calf serum**, 2mM L-Glutamine (Sigma, cat. no. G7513) or equivalent – for example, Glutamax (Invitrogen, cat. no. 35050-038) – and 1% penicillin/streptomycin (Invitrogen cat. no. 15140148).

[2] Example of an SOP for an established bioassay that may be adapted for use by laboratories developing secondary antibody standards.

- Dulbecco's MEM (Sigma, cat. no. D6546) or equivalent, supplemented with **4% fetal calf serum,** 2mM L-Glutamine (Sigma, cat. no. G7513) or equivalent – for example, Glutamax (Invitrogen, cat. no. 35050-038) – and 1% penicillin/streptomycin (Invitrogen, cat. no. 15140148).
- Trypsin/EDTA solution (Sigma, cat. no. T4049) or equivalent – for example, TrypLE Express (Invitrogen, cat. no. 12604-013).
- Dulbecco's MEM (Sigma, cat. no. D6546) or equivalent.
- Formaldehyde solution (Sigma, cat. no. 47673) prepared at 4% (v/v) in PBS-A (upon preparation, keep in fridge, for up to 2 months).
- Phosphate buffered saline (ThermoFisher, cat. no. 10010023).
- Washing buffer – PBS/0.05% (v/v) Tween 20 – alternatively prepare by adding Tween 20 (Sigma, cat. no. P1379) to PBS.
- 0.1% Triton-X100 (Sigma, cat. no. X100) diluted in PBS.
- Blocking buffer – washing buffer + 3% (w/v) Marvel milk powder.
- K-Blue aqueous TMB substrate (Neogen, cat. no. 331177).
- Stop solution – 2N $H_2SO_4$.

**Antibodies – pathogen specific**

Native Antigen Company: MAB12184-100-HRP or MAB12184-500-HRP, mouse anti-SARS-CoV-2 Np, horseradish peroxidase conjugated.

## Procedure

All the documents associated with this SOP must have been read and understood.

Ensure that the flask lid is closed while transporting it from the MSC to the incubator. Filtered lids are preferred and should be kept closed at all times in the incubator.

Plates should be transported to/from the MSC from/to incubator within sandwich boxes. Plates should be kept on a tray or in an open-lid sandwich box at all times in the incubator. Do not stack more than two plates on top of each other.

Wear thermal gloves when handling material at low temperatures (such as −80 °C and dry ice).

### *Day 1*

**This step can be performed in a biosafety level 2 (BSL2) or 3 (BSL3) laboratory**.

**If performed in a BSL3 laboratory**:

1. Turn on the MSC and ensure appropriate checks are performed.
2. Seed VERO cells (CCL-81) at 2 x $10^4$ cells per well in a 96-well flat-bottom plate to achieve confluent monolayers the next day.
3. Close the lids of the plates and place in a sealed container, spray with 70% IMS, remove outer gloves and take hands out of the MSC, re-glove and then remove sealed box from the MSC.
4. Place in a 37 ºC; 5% $CO_2$ incubator overnight, opening the box vent for gas exchange.

5. Alternatively, if this step is performed in a BSL2 laboratory, the plates will need to be transported to the BSL3 laboratory on the day of infection in a sealed container.

## *Day 2*

**Antibody dilutions can be performed in a BSL2 or BSL3 laboratory**.

If antibody dilutions have been undertaken in a BSL2 laboratory, the prepared plates are to be transported into the BSL3 laboratory in a sealed container.

All work with a live virus must be performed inside an MSC.

1. Turn on the BSL3 MSC and ensure appropriate checks are performed.
2. Collect the virus stock within a secondary container from storage and transport in the secondary container to the MSC.
3. Remove virus stock from container and place on a Wypall soaked with 70% IMS to defrost.
4. Check that the vial is defrosted and is not broken or leaking.
5. If the vial has leaked, then the sample should be disposed of as BSL3 waste.
6. Perform serial dilutions of the antibody samples in serum-free medium in a U-bottom 96-well plate, ideally in triplicate. Up to 4 antibody-containing samples can be assessed per plate (Fig. 1). An example of a dilution series is provided below with the relevant controls:

| | |
|---|---|
| Dilution 1 | 12 µL sample + 108 µL MEM (1/10 – final will be 1/20) |
| Dilution 2 | 60 µL dil 1 + 60 µL MEM |
| Dilution 3 | 60 µL dil 2 + 60 µL MEM |
| Dilution 4 | 60 µL dil 3 + 60 µL MEM |
| Dilution 5 | 60 µL dil 4 + 60 µL MEM |
| Dilution 6 | 60 µL dil 5 + 60 µL MEM, discard 60 µL |
| Positive Control | 60 µL MEM (virus only – no antibody) |
| Negative control | 120 µL MEM (no virus – cells only) |

Change tips between dilutions to avoid carryover.

**Fig. 1**
**Example of a 96-well plate layout for the titration of serum/plasma/antibody**

| Sample 1 | | | Sample 2 | | | Sample 3 | | | Sample 4 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | |
| | | | | | | | | | | | |
| | | | | | | | | | | | |
| | | | | | | | | | | | |
| | | | | | | | | | | | |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| CELLS ONLY | | | | | | VIRUS ONLY | | | | | |

7. Dilute the virus stock in medium without serum or antibiotics (for example, MEM or DMEM) to add 60 µL containing 100 $TCID_{50}$/well (for example, for a viral stock of 2 x $10^5$ $TCID_{50}$/mL – 100 ÷ 2 x $10^5$ = 0.5 µL virus stock per well and 50 µL virus stock diluted in 5950 µL serum-free medium to add 60 µL containing 100 $TCID_{50}$ to 100 wells).
8. To each antibody dilution and positive control add 60 µL of diluted virus prepared as above.
9. Close the lid of the plate and place in a sealed container, spray with 70% IMS, remove outer gloves and take hands out of MSC, re-glove and then remove sealed box from the MSC.
10. Place in the incubator at 37 ºC; 5% $CO_2$ for 1 hour.
11. Transfer plates with the virus/antibody dilutions and the plates seeded to the MSC within a sealed container.
12. Using a multichannel pipette, gently remove culture medium from the plates with seeded cells.
13. Transfer 100 µL of virus/antibody dilutions, and positive and negative controls, into each relevant well of the cell plate. An example of a potential layout is shown in Fig. 1.

The same tip can be used between replicates, but should be changed between dilutions to avoid carryover.

14. Label plates appropriately (virus name, antibody/sera name, date, dilution, user initials).
15. Using a multichannel pipette, add 100 µL of medium with 4% FCS in each well.
16. Close the lid of the plate and place in a sealed container, spray with IMS 70%, remove outer gloves and take hands out of MSC, re-glove and then remove sealed box from the MSC. Transport to the 37 ºC; 5% $CO_2$ incubator and open vent.
17. Incubate for 24 hours.

## *Day 3*

Performed in the afternoon, more than 24 hours post addition of the virus/Ab mix to the cells.

1. Turn on the BSL3 MSC and ensure appropriate checks are performed and recorded on log sheet prior to use.
2. Retrieve plates within sealed container from the incubator and transport to the MSC.
3. Using a multichannel pipette, remove culture medium from the plates, and wash cells with 200 µL PBS.
4. Add 200 µL of 4% formaldehyde solution in PBS to each well.
5. Close the lid of the plate and place in a sealed container, spray with IMS 70%, remove outer gloves and take hands out of MSC, re-glove and then remove sealed box from MSC. Transport to the fridge (4 ºC).

6. Incubate the plate for more than 16 hours.

### *Day 4*

1. Turn on the BSL3 MSC and ensure appropriate checks are performed.
2. Retrieve plates from fridge and transport to the MSC.
3. Remove the formaldehyde solution and wash once with 200 µL PBS.
4. Add 150 µL of 0.1% Triton-X100 (in PBS) to each well and incubate at room temperature for 15 minutes.
5. Remove with a multichannel pipette.
6. Wash plates once with 200 µL of PBS-Tween (0.05% v/v).
7. Add 200 µL of PBS-Tween (0.05% v/v)/3% milk (blocking buffer) to each well and block for 1 hour at room temperature inside the MSC.
8. Wash plates twice with 200 µL of PBS-Tween (0.05% v/v).
9. Add 50 µl/well of the relevant primary antibody diluted in blocking buffer (for example, anti-SARS-CoV-2 N protein diluted 1:2000).
10. Incubate plates for 1 hour at room temperature inside the MSC.
11. During the incubation, remove TMB substrate from the fridge and warm to room temperature, protected from light.
12. Wash plates 3 times with 200 µL of PBS-Tween (0.05% v/v).
13. Gently tap dry the plates on a Wypall.
14. Add 100 µl of TMB substrate per well, and incubate for 5–15 minutes.
15. Stop the reaction with 100 µL of 2N $H_2SO_4$.
16. Wipe the outside of the plate with a Wypall soaked in 70% IMS; leave the lid inside the MSC before transporting the plate to the reader.
17. Read plates at OD450 nm.

# Appendix 7

## Neutralization assay using SARS-CoV-2 spike lentiviral pseudotyped virus[3]

### Summary

Pseudotyped virus (PV)-based neutralization assays have been widely used as a surrogate for high-containment enveloped virus assays, allowing greater access to the study of virus-entry inhibition by different biologicals. In many cases, it has been shown that neutralization of the PV correlates with that of the corresponding virus, including in studies of SARS-CoV and MERS-CoV PVs *(1, 2)*. The system offers the advantages of being high throughput and quantitative, with results acquired 48 hours after assay set up through acquisition of reporter gene expression from target cells. This protocol describes a neutralization assay using a SARS-CoV-2 spike lentiviral PV incorporating a luciferase reporter gene, using HEK-293T clone 17 cells transiently expressing the cellular receptor ACE-2 and serin protease TMPRSS2 as the target cell line. The assay can be used to test the neutralizing activity of various biologicals such as serum, plasma and monoclonal antibodies.

The production of this SARS-CoV-2 lentiviral PV has been described elsewhere *(3, 4)*. In addition, other commonly used PV-based neutralization assays have also been developed, including one based on the use of a recombinant vesicular stomatitis virus (VSV) protocol for the production of the SARS-CoV-2-PV and neutralization assay *(5)*.

### Materials

#### Cell lines

- HEK-293T clone 17 cells (NIBSC CFAR, cat. no. 5016).

#### Cell culture medium

HEK-293T clone 17 cells

- Gibco DMEM (1X) + GlutaMAX (ThermoFisher, cat. no. 61965-026);
- 10% v/v fetal calf serum (Pan Biotech GmbH, cat. no. P30-3306, heat inactivated, South American origin); and
- 1% v/v penicillin–streptomycin (Sigma-Aldrich, cat. no. P0781).

#### Plasmids/recombinant virus

- Expression plasmid: pCDNA3.1 hACE2 (Addgene, cat. no. 1786)
- Expression plasmid: pCSDest TMPRSS2 (Addgene, cat. no. 53887).

#### Reagents

- 0.25% trypsin-EDTA solution (Sigma-Aldrich, cat. no. T4049)
- Gibco Opti-MEM I (1X) (ThermoFisher, cat. no. 31985-047)
- Gibco DMEM (1X) phenol free (ThermoFisher, cat. no. 31053-028)
- FuGENE HD transfection reagent (Promega, cat. no. E2311)

[3] Example of an SOP for an established bioassay that may be adapted for use by laboratories developing secondary antibody standards.

- Bright-Glo® luciferase assay system (Promega, cat. no. E2620).

**Consumables/equipment**

- 10 cm TC-treated culture dish (Corning, cat. no. 430167);
- Falcon MicroWell TC-treated flat-bottom 96-well plate (ThermoFisher, cat. no. 10334791);
- Falcon MicroWell TC-treated U-bottom 96-well plate (ThermoFisher, cat. no. 10520832);
- Nunc F96 MicroWell white 96-well microplate (ThermoFisher, cat. no. 236108);
- Polypropylene sterile conical tubes, 15 mL (Sarstedt, cat. no. 62.554.502);
- 1.5 mL sterile micro-tubes (Sarstedt, cat. no. 72.692.005);
- GloMax® navigator microplate luminometer, or similar (Promega, cat. no. GM2000); and
- Incubator at 37 °C; 5% $CO_2$.

## Procedure

**All work must be carried out inside an MSC in a BSL2 laboratory, following local risk assessments and guidance on working with genetically modified microorganisms based on non-replicative lentiviral vectors**.

### *Day 1: Seed target cells in preparation for transfection*

1. Seed a 10 cm culture dish with 5 x $10^6$ HEK-293T/17 cells in 8 mL culture medium, to reach 60–80% confluence the next day. Typically, a single 10 cm dish yields enough cells for at least 7 x 96-well assay plates on Day 3 – seed more plates as required.
2. Incubate overnight at 37 °C; 5% $CO_2$.

### *Day 2: Target cell transfection with receptor and protease expression plasmids*

1. Pre-warm to ambient temperature culture medium for HEK-293T/17 cells, Opti-MEM and FuGENE HD.
2. Prepare a sterile 1.5 mL micro-tube containing the following quantity of plasmid for transfection:
   - 2 μg pCDNA3.1 hACE2
   - 150 ng pCSDest TMPRSS2.
3. Add 200 μL Opti-MEM to the tube containing plasmid, briefly vortex to mix and pulse centrifuge.
4. Next, add directly into the centre 6.5 μL of FuGENE HD transfection reagent (3:1 volume to mass ratio), gently flick to mix 3–4 times.
5. Incubate inside the MSC for 10–15 minutes.
6. During incubation, gently replace 8 mL culture medium of the HEK-293T/17 cells seeded into a culture dish the previous day.
7. Following incubation, add the transfection mix drop-wise to the cell culture dish while gently agitating plate to ensure even dispersal.
8. Incubate at 37 °C; 5% $CO_2$ for 24 hours.

### *Day 3: Neutralization of SARS-CoV-2 spike lentiviral pseudotyped virus*

1. Following 24 hours incubation, remove medium from transfected target cells and detach from surface by incubation with 0.25% trypsin-EDTA or by following standard laboratory protocol for re-suspension of adherent cell lines.
2. Count cells and dilute with culture medium to 2 x $10^5$ cells/mL.
3. Add 100 µL per well of a 96-well microplate to give 2 x $10^4$ cells/well.
4. Incubate at 37 °C; 5% $CO_2$ for a minimum of 2 hours.
5. If frozen, retrieve test samples and thaw at ambient temperature. It is recommended to include a positive control sample with known neutralizing activity and appropriate negative control sample each time an assay is performed.
6. Calculate the amount of SARS-CoV-2 PV required; it is recommended to use an input of 150–300 $TCID_{50}$/well.
7. Retrieve from −80 °C storage the required number of aliquots of the SARS-CoV-2 PV and thaw at ambient temperature.
8. In a 96-well sterile U-bottom plate, prepare a dilution series of each test sample, and positive and negative control samples, within a final volume of 60 µL culture medium, taking into account the 1:2 dilution after the addition of PV at step 9. A threefold dilution series is recommended performed at least in triplicate. Each plate should also contain control wells of cells only and PV only.

As per the example layout shown in Fig. 1, 81 µL culture medium is added into row A and 60 µL into all remaining wells. Next, 9 µL serum is added into each well of row A before performing a threefold serial dilution by carrying 30 µL across the rest of the dilution series rows B–G, discarding the final 30 µL.

**Fig. 1**
**Example of a 96-well plate layout for PV neutralization assay**

| | **Sample 1** | | | **Sample 2** | | | **Sample 3** | | | **Sample 4** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 1/20 | 1/20 | 1/20 | 1/20 | 1/20 | 1/20 | 1/20 | 1/20 | 1/20 | 1/20 | 1/20 | 1/20 |
| B | 1/60 | 1/60 | 1/60 | 1/60 | 1/60 | 1/60 | 1/60 | 1/60 | 1/60 | 1/60 | 1/60 | 1/60 |
| C | 1/180 | 1/180 | 1/180 | 1/180 | 1/180 | 1/180 | 1/180 | 1/180 | 1/180 | 1/180 | 1/180 | 1/180 |
| D | 1/540 | 1/540 | 1/540 | 1/540 | 1/540 | 1/540 | 1/540 | 1/540 | 1/540 | 1/540 | 1/540 | 1/540 |
| E | 1/1620 | 1/1620 | 1/1620 | 1/1620 | 1/1620 | 1/1620 | 1/1620 | 1/1620 | 1/1620 | 1/1620 | 1/1620 | 1/1620 |
| F | 1/4860 | 1/4860 | 1/4860 | 1/4860 | 1/4860 | 1/4860 | 1/4860 | 1/4860 | 1/4860 | 1/4860 | 1/4860 | 1/4860 |
| G | 1/14580 | 1/14580 | 1/14580 | 1/14580 | 1/14580 | 1/14580 | 1/14580 | 1/14580 | 1/14580 | 1/14580 | 1/14580 | 1/14580 |
| H | CELLS ONLY | | | | | | PV ONLY | | | | | |

9. Dilute SARS-CoV-2 PV in culture medium to add 60 µL containing 150–300 $TCID_{50}$ to each well of the dilution plate except the cell-only controls, to which 60 µL of culture medium only should be added to each well.

10. Incubate at 37 °C for 30–60 minutes.
11. Transfer 100 µL from each well of the dilution plate to the 96-well culture plate seeded with target cells as described in step 3 above (> 2 hours earlier).
12. Incubate at 37 °C; 5% $CO_2$ for 48 or 60 hours.

### *Day 5 or 6: Acquisition of results and data analysis*

1. Following the 48 or 60 hours incubation period, prepare the Bright-Glo® reagent by reconstituting the Bright-Glo® substrate (brown glass bottle) with addition of the Bright-Glo® buffer (white bottle). Mix by inversion until the substrate is thoroughly dissolved. Aliquot and store the reconstituted reagent at between −77 °C and −83 °C for up to 1 year. Thaw the Bright-Glo® reagent at temperatures below 25 °C, equilibrate to room temperature and mix well before use.
2. Retrieve 96-well culture plates from the incubator and remove the supernatant without disturbing the cells.
3. Add 100 µL of a 1:1 mix of phenol-free DMEM and Bright-Glo® reagent to each well and incubate for 5 minutes (± 2 minutes) at a room temperature of 24 °C (± 4 °C) to allow cell lysis.
4. Gently mix each well, by pipetting up and down once, before transferring 90 µL of the mixture to a 96-well white plate in the same format.
5. Read the plate on the GloMax® navigator microplate luminometer, or similar equipment.
6. To determine the half maximal inhibitory concentration ($IC_{50}$) of the test samples, normalize the raw data to express results as % neutralization by defining 100% neutralization as the mean of the cell-only wells and 0% neutralization as the mean of the PV-only wells.
7. Plot a graph of the average % neutralization (y-axis) against the $\log_{10}$ sample dilution (x-axis).
8. Fit a dose-dependent inhibition curve to the data via nonlinear regression analysis to interpolate the $IC_{50}$ values. It is recommended that this analysis is performed using software such as GraphPad Prism® and associated published detailed protocol *(6)*.

## References


1. Temperton NJ, Chan PK, Simmons G, Zambon MC, Tedder RS, Takeuchi Y et al. Longitudinally profiling neutralizing antibody response to SARS coronavirus with pseudotypes. Emerg Infect Dis. 2005;11(3):411–6 (https://wwwnc.cdc.gov/eid/article/11/3/04-0906_article, accessed 23 April 2022).

2. Perera RA, Wang P, Gomaa MR, El-Shesheny R, Kandeil A, Bagato O et al. Seroepidemiology for MERS coronavirus using microneutralisation and pseudoparticle virus neutralisation assays reveal a high prevalence of antibody in dromedary camels in Egypt, June 2013. Euro Surveill. 2013;18(36) (https://www.eurosurveillance.org/content/10.2807/1560-7917.ES2013.18.36.20574?msclkid=ba7da201c31711ecb9c463d1cf1b8003, accessed 23 April 2022).

3. Di Genova C, Sampson A, Scott S, Cantoni D, Mayora-Neto M, Bentley E et al. Production, titration, neutralisation, storage and lyophilisation of severe acute respiratory

syndrome coronavirus 2 (SARS-CoV-2) lentiviral pseudotypes. Bio Protoc. 2021;11(21):e4236 (https://bio-protocol.org/e4236, accessed 23 April 2022).

4. Mekkaoui L, Bentley EM, Ferrari M, Lamb K, Ward K, Karattil R et al. Optimised Method for the production and titration of lentiviral vectors pseudotyped with the SARS-CoV-2 spike. Bio Protoc. 2021;11(16):e4194 (https://bio-protocol.org/bio101/e4034, accessed 23 April 2022).

5. Nie J, Li Q, Wu J, Zhao C, Hao H, Liu H et al. Quantification of SARS-CoV-2 neutralizing antibody by a pseudotyped virus-based assay. Nat Protoc. 2020;15:3699–715 (https://www.nature.com/articles/s41596-020-0394-5, accessed 23 April 2022).

6. Ferrara F, Temperton N. Pseudotype neutralization assays: from laboratory bench to data analysis. Methods Protoc. 2018;1(1):8 (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6526431/?msclkid=9af53b9bc31211eca4d5c4afdd9c679f, accessed 23 April 2022).

# Appendix 8

## Calibrating SARS-CoV-2 immunoassay internal assay reference reagents to international standards and/or secondary standards[4]

### Purpose

This appendix describes the procedure for using international standards and/or secondary standards to calibrate internal assay reference reagents for SARS-CoV-2 immunoassays.

### Scope

This guidance applies to all SARS-CoV-2 immunoassays requiring the calibration of internal assay reference reagents to an international standard and/or secondary standard.

### Definitions

- **Calibrator**: biological material, such as antibodies, found in nature and which has a reference value assigned.
- **WHO IS**: World Health Organization International Standard.
- **Primary standard**: biological substance, which is provided to the global community to enable harmonization by expressing results from a biological assay or immunological assay in the same way throughout the world.
- **Secondary standard**: reference standards established by regional or national authorities, or by other laboratories, that are calibrated against, and traceable to, the primary WHO materials and are intended for use in routine tests.

### Procedure principles

Assign an International Unit per millilitre (IU/mL) or binding antibody unit per millilitre (BAU/mL) to an internal assay reference reagent that is used daily. The unit will be dependent upon the type of calibrator being used – an international standard (primary standard) will have an assigned IU, while a secondary standard will have another unit assigned (such as arbitrary units, mg or index value) unless it has been calibrated to the international standard – in which case, the units of the secondary standard will be IU/mL.

### Procedure

Test the calibrator (WHO IS or secondary standard when a WHO IS is not available) in triplicate (independent serial dilutions) in the same plate as the internal assay reference reagent (daily assay standard). Perform serial dilutions of the calibrator so that the calibrator reaches end-point dilution/titre/concentration. For consistency, the fold dilution of the calibrator should match the fold dilution of the internal assay reference reagent (for example, twofold or threefold serial dilution).

Fig. 1 shows a representative plate map design for an immunoassay – alternative schemes may be used to suit a specific assay. As shown in Fig. 1, serial dilutions of each sample should be used and each sample tested at least in triplicate. Assay controls per standard

[4] Example of an SOP for an established bioassay that may be adapted for use by laboratories developing secondary antibody standards.

operating procedure should be included in each plate to verify system suitability. The test should also be performed on 3 separate days in the exact same manner and set-up used on Day 1. Of note, a new vial of calibrator and internal assay reference reagent, which has not gone through freeze-thaw events, should be used on each day of testing. Depending on availability, the plate map includes space to test an additional secondary standard, which will allow for the simultaneous calibration of a secondary standard and internal assay reference reagent.

**Fig. 1**
**Plate map of a calibration set up**

| Day 1 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Plate 1 | C_STD | C_STD | NEG | PC1 | STD-C1 | STD-C2 | STD-C3 | STD-T1 | STD-T2 | STD-T3 | C_STD | C_STD |
| A | 50 | 50 | 50 | 50 | 200 | 200 | 200 | 200 | 200 | 200 | 50 | 50 |
| B | 100 | 100 | 150 | 150 | 400 | 400 | 400 | 400 | 400 | 400 | 100 | 100 |
| C | 200 | 200 | 450 | 450 | 800 | 800 | 800 | 800 | 800 | 800 | 200 | 200 |
| D | 400 | 400 | 1350 | 1350 | 1600 | 1600 | 1600 | 1600 | 1600 | 1600 | 400 | 400 |
| | | | No Sample | PC2 | | | | | | | | |
| E | 800 | 800 | 50 | 150 | 3200 | 3200 | 3200 | 3200 | 3200 | 3200 | 800 | 800 |
| F | 1600 | 1600 | 150 | 450 | 6400 | 6400 | 6400 | 6400 | 6400 | 6400 | 1600 | 1600 |
| G | 3200 | 3200 | 450 | 1350 | 12800 | 12800 | 12800 | 12800 | 12800 | 12800 | 3200 | 3200 |
| H | 6400 | 6400 | 1350 | 4050 | 25600 | 25600 | 25600 | 25600 | 25600 | 25600 | 6400 | 6400 |

C_STD = internal assay reference reagent; STD-C1, C2 and C3 = calibrator; STD-T1, T2 and T3 = secondary standard; NEG = negative control; PC = positive control.

## Data analysis

First, it is recommended to test for parallelism between the dose–response curve of the calibrator and the dose–response curve of the internal assay reference reagent. Molecular Devices (SoftMax Pro 6.5+) and CombiStats™ are two commercial off-the-shelf programmes that can perform parallel line analysis, and the analysis can also be completed in R. Parallelism methods can be grouped into two categories – response comparison tests and parameter comparison tests. A chi-square test of the extra-sum-of-squares statistic is recommended to test for parallelism as it generally provides an estimate of the dose–response curves with the least amount of bias.

The calibrator is treated as the reference, and the potency value for the calibrator may be found in the respective Instructions for Use (IFU) document. For reference, Table 1 shows the unitages of the First WHO International Standard for anti-SARS-CoV-2 immunoglobulin (NIBSC code 20/136)[5] when reconstituted in accordance with the IFU.

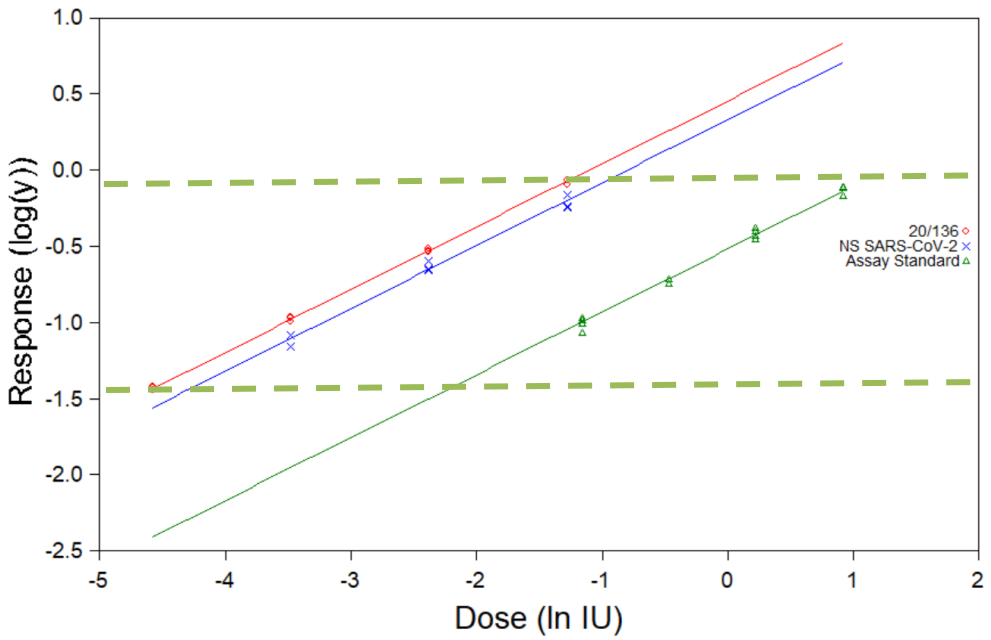
**Table 1**
**Assigned neutralizing and binding unitages of the First WHO International Standard for anti-SARS-CoV-2 immunoglobulin (NIBSC code 20/136)**

[5] Following rapid depletion of stocks of this WHO IS, the Second WHO International Standard for anti-SARS-CoV-2 immunoglobulin will be proposed for establishment in 2022.

| | First WHO IS for anti-SARS-CoV-2 immunoglobulin (NIBSC code 20/136) |
|---|---|
| Neutralizing assays | 1000 IU/mL |
| IgM (spike) | 1000 BAU/mL |
| IgM (nucleocapsid) | 1000 BAU/mL |
| IgG (spike) | 1000 BAU/mL |
| IgG (nucleocapsid) | 1000 BAU/mL |

Fig. 2 shows the dose (ln IU) versus response (log(y)) curves for: (a) the First WHO International Standard for anti-SARS-CoV-2 immunoglobulin (red line); (b) a hypothetical national SARS-CoV-2 serology standard (blue line); and (c) a hypothetical internal assay reference reagent (green line). When reviewing such plots, it should be verified that the response values of the internal assay reference reagent and other reagents (such as the secondary standard) fall within the response range of the calibrator (see grey dotted lines in Fig. 2) to avoid performing analysis on extrapolated data. CombiStats™ allows the analyst to assign a relative potency value to the calibrator, and the programme will perform the relevant calculations needed to determine if the national serology standard and internal assay reference reagent samples are parallel to the calibrator – as depicted in Fig. 2. The analyst can then review the probability of the dose (ln IU) versus response (log(y)) lines being non-parallel and non-linear. A probability value greater than 0.05 for non-parallelism and non-linearity will indicate that the dose–response lines are parallel and linear. Furthermore, the CombiStats™ programme can calculate the relative potency of the samples, and this value in turn can be used to calculate the potency of the sample across the 3 days of testing.

**Fig. 2**
**Parallelism graph for spike IgG assay using CombiStats™**




***Note***: the data shown in Fig. 2 are hypothetical and intended only for illustration purposes.

The estimated potency value calculated from the dose–response curve generated from each replicate series of serial dilutions for each sample is averaged for each day of testing. Finally,

the geometric mean of the estimated potency values from each of the 3 days is calculated and will represent the final potency (calibrated) value for each sample. Table 2 illustrates the calibration process based on representative CombiStats™ data.

**Table 2**
**Representative CombiStats™ data illustrating the calibration process using a quantitative assay**

| Sample ID | Mean Day 1 | Mean Day 2 | Mean Day 3 | Geometric mean |
|---|---|---|---|---|
| STD-C | 1000 | 1000 | 1000 | 1000 |
| STD-T | 694 | 769 | 743 | 735 |
| C_STD | 92 | 97 | 90 | 93 |

STD-C = calibrator; STD-T = secondary standard; C_STD = internal assay reference reagent.

***Note***: the data shown in Table 2 are hypothetical and intended only for illustration purposes.

**Calibration calculations**

***Assumptions***: STD-C = 1000 BAU/mL; STD-T = 735 BAU/mL; C_STD = 93 BAU/mL.

Unfortunately, the calibration process is not uniform for all immunoassays. For example, in the case of semi-quantitative assays (such as neutralization assays), parallelism is difficult to calculate due to the assay methodology. In this circumstance, the following procedure will be applicable. Although neutralization assays are set up using serial dilution of the sample, with each sample typically tested in multiple replicates (such as in triplicate), the readout of the assay may not utilize a linear or logistic curve to determine a titre. These types of assays may be calibrated by calculating the mean of the titre (reciprocal of the last dilution indicating 100% neutralization) from the triplicate tests for each day, and then the geometric mean of the averaged results from Day 1, Day 2 and Day 3 are calculated. The geometric mean value is then treated as the final value. Table 3 illustrates the calibration process for each sample evaluated in a semi-quantitative assay.

**Table 3**
**Representative data to illustrate the calibration process using a semi-quantitative assay**

| SID | 100% Neut Day 1* | 100% Neut Day 2* | 100% Neut Day 3* | Mean Day 1 | Mean Day 2 | Mean Day 3 | Geometric Mean |
|---|---|---|---|---|---|---|---|
| STD-C1 | 800 | 1600 | 800 | 1067 | 1067 | 800 | 969 |
| STD-C2 | 1600 | 800 | 800 | | | | |
| STD-C3 | 800 | 800 | 800 | | | | |
| STD-T1 | 400 | 400 | 800 | 667 | 400 | 533 | 522 |
| STD-T2 | 800 | 400 | 400 | | | | |
| STD-T3 | 800 | 400 | 400 | | | | |
| C_STD | 3200 | 1600 | 1600 | 2667 | 3733 | 2667 | 2983 |
| C_STD | 3200 | 6400 | 3200 | | | | |
| C-STD | 1600 | 3200 | 3200 | | | | |

STD-C1, C2 and C3 = calibrator; STD-T1, T2 and T3 = secondary standard; C_STD = internal assay reference reagent.

***Note***: the data shown in Table 3 are hypothetical and intended only for illustration purposes.

**Calibration calculations**

***Assumptions***:

STD-C (calibrator) = 1000 IU/mL
STD-T (1000 IU/mL/969 titre) x 522 titre = 539 IU/mL
C_STD (1000 IU/mL/969 titre) x 2983 titre = 3078 IU/mL

***Note***: the final calibration value will be dependent upon the reporting system established within the laboratory, such as rounding up the nearest dilution (titre) or if the laboratory uses a continuous model to calculate titres for each sample.

## Further reading


1. Recommendations for the preparation, characterization and establishment of international and other biological reference standards (revised 2004). In: WHO Expert Committee on Biological Standardization: fifty-fifth report. Geneva: World Health Organization; 2006: Annex 2 (WHO Technical Report Series, No. 932; https://www.who.int/publications/m/item/annex2-trs932?msclkid=69b33360c3e811ec9b73e5f303acf225, accessed 24 April 2022).
2. Application Note, Parallel line analysis and relative potency in SoftMax Pro 7 Software, 2016 Molecular Devices, LLC.
3. Gottschalk PD, Dunn JR. Measuring parallelism, linearity, and relative potency in bioassay and immunoassay data. J Biopharm Stats. 2005;15(3):437–63 (abstract: https://www.tandfonline.com/doi/abs/10.1081/BIP-200056532?journalCode=lbps20, accessed 24 April 2022).
4. Bates DM, Watts DG. Nonlinear regression analysis and its applications. New York: John Wiley & Sons, Inc.; 1988 (available from: https://onlinelibrary.wiley.com/doi/book/10.1002/9780470316757?msclkid=3a7e8630c3e911ecb48ce258f89aec9d, accessed 24 April 2022).

# Appendix 9

## Calibrating human papillomavirus (HPV) immunoassay internal assay reference reagents to international standards and/or secondary standards[6]

### Purpose

This appendix describes the procedure for using international standards and/or secondary standards to calibrate internal assay reference reagents for human papillomavirus (HPV) immunoassays.

### Scope

This guidance applies to all HPV immunoassays requiring the calibration of internal assay reference reagents to an international standard and/or secondary standard.

### Definitions

- **Calibrator**: biological material, such as antibodies, found in nature and which has a reference value assigned.
- **WHO IS**: World Health Organization International Standard.
- **Primary standard**: biological substance, which is provided to the global community to enable harmonization by expressing results from a biological assay or immunological assay in the same way throughout the world.
- **Secondary standard**: reference standards established by regional or national authorities, or by other laboratories, that are calibrated against, and traceable to, the primary WHO materials and are intended for use in routine tests.

### Procedure principles

Assign an International Unit per millilitre (IU/mL) or binding antibody unit per millilitre (BAU/mL) to an internal assay reference reagent that is used daily. The unit will be dependent upon the type of calibrator being used – an international standard (primary standard) will have an assigned IU, while a secondary standard will have another unit assigned (such as arbitrary units, mg or index value) unless it has been calibrated to the international standard – in which case, the units of the secondary standard will be IU/mL.

### Procedure

Test the calibrator (WHO IS or secondary standard when a WHO IS is not available) in triplicate (independent serial dilutions) in the same plate as the internal assay reference reagent (daily assay standard). Perform serial dilutions of the calibrator, so the calibrator reaches end-point dilution/titre/concentration. For consistency, the fold dilution of the calibrator should match the fold dilution of the internal assay reference reagent (for example, twofold or threefold serial dilution).

Fig. 1 shows a representative plate map design for an immunoassay – alternative schemes may be used to suit a specific assay. As shown in Fig. 1, serial dilutions of each sample

[6] Example of an SOP for an established bioassay that may be adapted for use by laboratories developing secondary antibody standards.

should be used and each sample tested at least in triplicate. Assay controls per standard operating procedure should be included in each plate to verify system suitability. The test should also be performed on 3 separate days in the exact same manner and set-up used on Day 1. Of note, a new vial of calibrator and internal assay reference reagent, which has not gone through freeze-thaw events, should be used on each day of testing. Depending on availability, the plate map includes space to test an additional secondary standard, which will allow for the simultaneous calibration of a secondary standard and internal assay reference reagent.

**Fig. 1**
**Plate map of a calibration set up**

| Day 1 Plate 1 | 1 C_STD | 2 C_STD | 3 NEG | 4 PC1 | 5 STD-C1 | 6 STD-C2 | 7 STD-C3 | 8 STD-T1 | 9 STD-T2 | 10 STD-T3 | 11 C_STD | 12 C_STD |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 50 | 50 | 50 | 50 | 200 | 200 | 200 | 200 | 200 | 200 | 50 | 50 |
| B | 100 | 100 | 150 | 150 | 400 | 400 | 400 | 400 | 400 | 400 | 100 | 100 |
| C | 200 | 200 | 450 | 450 | 800 | 800 | 800 | 800 | 800 | 800 | 200 | 200 |
| D | 400 | 400 | 1350 | 1350 | 1600 | 1600 | 1600 | 1600 | 1600 | 1600 | 400 | 400 |
| | | | No Sample | PC2 | | | | | | | | |
| E | 800 | 800 | 50 | 150 | 3200 | 3200 | 3200 | 3200 | 3200 | 3200 | 800 | 800 |
| F | 1600 | 1600 | 150 | 450 | 6400 | 6400 | 6400 | 6400 | 6400 | 6400 | 1600 | 1600 |
| G | 3200 | 3200 | 450 | 1350 | 12800 | 12800 | 12800 | 12800 | 12800 | 12800 | 3200 | 3200 |
| H | 6400 | 6400 | 1350 | 4050 | 25600 | 25600 | 25600 | 25600 | 25600 | 25600 | 6400 | 6400 |

C_STD = internal assay reference reagent; STD-C1, C2 and C3 = calibrator; STD-T1, T2 and T3 = secondary standard; NEG = negative control; PC = positive control.

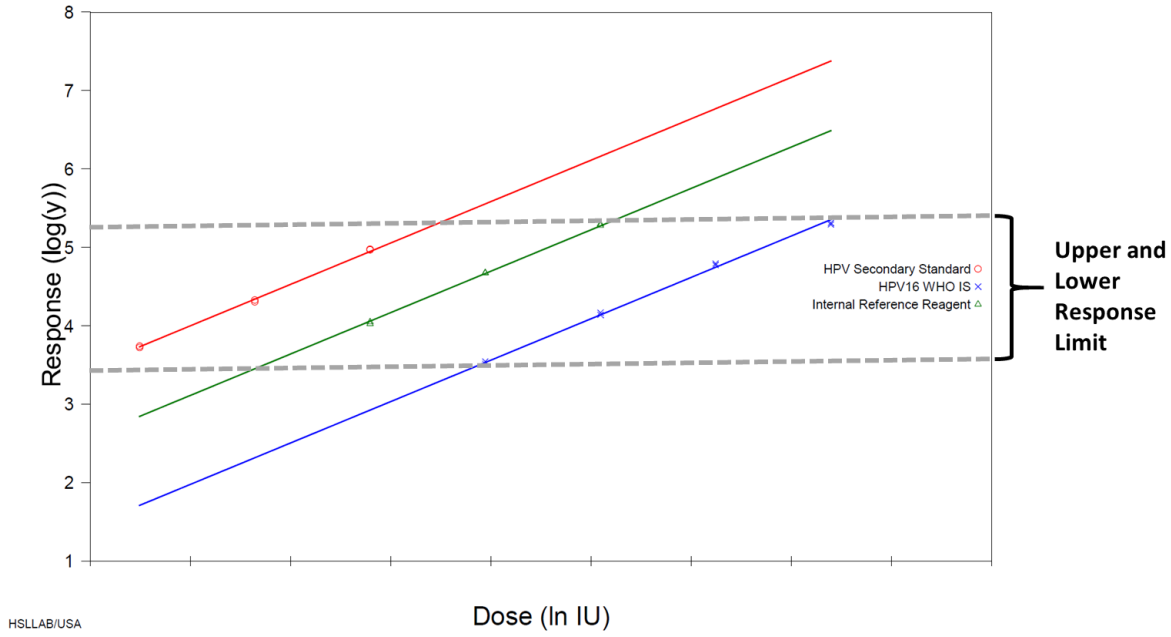
## Data analysis

First, it is recommended to test for parallelism between the dose–response curve of the calibrator and the dose–response curve of the internal assay reference reagent. Molecular Devices (SoftMax Pro 6.5+) and CombiStats™ are two commercial off-the-shelf programmes that can perform parallel line analysis, and the analysis can also be completed in R. Parallelism methods can be grouped into two categories – response comparison tests and parameter comparison tests. A chi-square test of the extra-sum-of-squares statistic is recommended to test for parallelism as it generally provides an estimate of the dose–response curves with the least amount of bias.

The calibrator is treated as the reference, and the potency value for the calibrator may be found in the respective Instructions for Use (IFU) document. Fig. 2 shows the dose (ln IU) versus response (log(y)) for: (a) the First WHO International Standard for antibodies to human papillomavirus type 16 (NIBSC code 05/134) (blue line); (b) a hypothetical HPV secondary standard (red line); and (c) a hypothetical internal assay reference reagent (green line). When reviewing such plots, it should be verified that the response values of the internal assay reference reagent and other reagents (such as the secondary standard) fall within the response range of the calibrator (see grey dotted lines in Fig. 2) to avoid performing analysis on extrapolated data. CombiStats™ allows the analyst to assign a relative potency value to the calibrator, and the programme will perform the relevant calculations needed to determine if the secondary standard and internal assay reference reagent are parallel to the calibrator – as depicted in Fig. 2. The analyst can then review the probability of the dose (ln IU) versus response (log(y)) lines being non-parallel and non-linear. A probability value greater than 0.05 for non-parallelism and non-linearity will indicate that the dose–response lines are

parallel and linear. Furthermore, the CombiStats™ programme can calculate the relative potency of the samples, and this value in turn can be used to calculate the potency of the sample across the 3 days of testing.

**Fig. 2**
**Parallelism graph for anti-HPV-16 IgG assay using CombiStats™**




***Note***: the data shown in Fig. 2 are hypothetical and intended only for illustration purposes.

The estimated potency value calculated from the dose–response curve generated from each replicate series of serial dilutions for each sample is averaged for each day of testing. Finally, the geometric mean of the estimated potency values from each of the 3 days is calculated and will represent the final potency (calibrated) value for each sample. Table 1 illustrates the calibration process based on representative CombiStatsTM data.

**Table 1**
**Representative CombiStats™ data illustrating the calibration process using a quantitative assay**

| Sample ID | Mean Day 1 | Mean Day 2 | Mean Day 3 | Geometric mean |
|---|---|---|---|---|
| STD-C | 10 | 10 | 10 | 10 |
| STD-T | 694 | 769 | 743 | 735 |
| C_STD | 92 | 97 | 90 | 93 |

STD-C = calibrator; STD-T = secondary standard; C_STD = internal assay reference reagent.

***Note***: The data shown in Table 1 are hypothetical and intended only for illustration purposes.

**Calibration calculations**

***Assumptions***: STD-C = 10 IU/mL; STD-T = 735 IU/mL; C_STD = 93 IU/mL.

Unfortunately, the calibration process is not uniform for all immunoassays. For example, in the case of semi-quantitative assays (such as neutralization assays) parallelism is difficult to calculate due to the assay methodology. In this circumstance, the following procedure will be applicable. Although neutralization assays are set up using serial dilution of the sample, with each sample typically tested in multiple replicates (such as in triplicate), the readout of the assay may not utilize a linear or logistic curve to determine a titre. These types of assays may be calibrated by calculating the mean of the titre (reciprocal of the last dilution indicating 100% neutralization) from the triplicate tests for each day, and then the geometric mean of the averaged results from Day 1, Day 2 and Day 3 are calculated. The geometric mean value is then treated as the final value. Table 2 illustrates the calibration process for each sample evaluated in a semi-quantitative assay.

**Table 2**
**Representative data to illustrate the calibration process using a semi-quantitative assay**

| SID | 100% Neut Day 1* | 100% Neut Day 2* | 100% Neut Day 3* | Mean Day 1 | Mean Day 2 | Mean Day 3 | Geometric Mean |
|---|---|---|---|---|---|---|---|
| STD-C1 | 800 | 1600 | 800 | 1067 | 1067 | 800 | 969 |
| STD-C2 | 1600 | 800 | 800 | | | | |
| STD-C3 | 800 | 800 | 800 | | | | |
| STD-T1 | 400 | 400 | 800 | 667 | 400 | 533 | 522 |
| STD-T2 | 800 | 400 | 400 | | | | |
| STD-T3 | 800 | 400 | 400 | | | | |
| C_STD | 3200 | 1600 | 1600 | 2667 | 3733 | 2667 | 2983 |
| C_STD | 3200 | 6400 | 3200 | | | | |
| C-STD | 1600 | 3200 | 3200 | | | | |

STD-C1, C2 and C3 = calibrator; STD-T1, T2 and T3 = secondary standard; C_STD = internal assay reference reagent.

***Note***: the data shown in Table 2 are hypothetical and intended only for illustration purposes.

**Calibration calculations**

***Assumptions***:

STD-C (calibrator) = 1000 IU/mL
STD-T (1000 IU/mL/969 titre) x 522 titre = 539 IU/mL
C_STD (1000 IU/mL/969 titre) x 2983 titre = 3078 IU/mL

***Note***: the final calibration value will be dependent upon the reporting system established within the laboratory, such as rounding up the nearest dilution (titre) or if the laboratory uses a continuous model to calculate titres for each sample.

## Further reading


1. Recommendations for the preparation, characterization and establishment of international and other biological reference standards (revised 2004). In: WHO Expert Committee on Biological Standardization: fifty-fifth report. Geneva: World Health Organization; 2006: Annex 2 (WHO Technical Report Series, No. 932; https://www.who.int/publications/m/item/annex2-trs932?msclkid=69b33360c3e811ec9b73e5f303acf225, accessed 24 April 2022).
2. Application Note, Parallel line analysis and relative potency in SoftMax Pro 7 Software, 2016 Molecular Devices, LLC.
3. Gottschalk PD, Dunn JR. Measuring parallelism, linearity, and relative potency in bioassay and immunoassay data. J Biopharm Stats. 2005;15(3):437–63 (abstract: https://www.tandfonline.com/doi/abs/10.1081/BIP-200056532?journalCode=lbps20, accessed 24 April 2022).
4. Bates DM, Watts DG. Nonlinear regression analysis and its applications. New York: John Wiley & Sons, Inc.; 1988 (available from: https://onlinelibrary.wiley.com/doi/book/10.1002/9780470316757?msclkid=3a7e8630c3e911ecb48ce258f89aec9d, accessed 24 April 2022).

# Appendix 10

## Standardization of respiratory syncytial virus (RSV) neutralization assays[7]

The neutralization assay is a widely used method for measuring neutralizing antibody titres against respiratory syncytial virus (RSV). The classical method used is the plaque reduction neutralization test (PRNT). However, the PRNT is a labour-intensive, lengthy and relatively low-throughput method. Individual laboratories have therefore created a diverse array of RSV neutralization assay formats that provide faster and higher-throughput alternatives to the PRNT. This diversity makes it difficult to compare RSV neutralizing antibody results across studies, and for different candidate RSV vaccines. The use of a common reference standard is an essential step towards reducing the inter-assay and inter-laboratory variability of RSV neutralization titre results.

WHO standardization activities coordinated by the National institute for Biological Standardization and Control (NIBSC) led to the development and establishment of the First WHO International Standard for antiserum to respiratory syncytial virus. The results of two international collaborative studies *(1, 2)* indicated that two candidate materials (NIBSC codes 16/284 and 16/322) evaluated in both studies were commutable with human sera samples. However, neither material was commutable with the animal sera samples or monoclonal antibodies tested. Study results further indicated that inter-laboratory variability in neutralization titres was substantially reduced when values were expressed relative to either of the candidate materials. Based on these results, candidate material 16/284 was established as the First WHO International Standard for antiserum to respiratory syncytial virus, with an assigned unitage of 1000 International Units (IU) of both anti-RSV/A and anti-RSV/B neutralizing antibodies per ampoule, by the WHO Expert Committee on Biological Standardization, with 16/322 considered to be suitable as a potential replacement standard for 16/284 (Table 1).

**Table 1**
**WHO IS for RSV**

| Reference standard | Source | NIBSC code[a] | Unitage (IU/ampoule)[b] |
|---|---|---|---|
| First WHO International Standard for antiserum to respiratory syncytial virus | NIBSC | 16/284 | RSV/A: 1000 IU/ampoule<br>RSV/B: 1000 IU/ampoule |
| Potential future replacement Second WHO International Standard for antiserum to respiratory syncytial virus | NIBSC | 16/322 | RSV/A: 960 IU/ampoule<br>RSV/B: 690 IU/ampoule |

[a] Both 16/284 and 16/322 consist of serum obtained from human adults who were all seropositive for RSV.
[b] Each ampoule contains 0.5 mL of freeze-dried human serum.

[7] Example of an SOP for an established bioassay that may be adapted for use by laboratories developing secondary antibody standards.

WHO encourages the use of the First WHO International Standard for antiserum to respiratory syncytial virus (NIBSC code 16/284) as it has been shown to substantially reduce both the inter-assay and inter-laboratory variability of antibody titres against RSV/A and RSV/B in human sera *(1, 2, 3)*. This WHO IS is therefore a primary standard of critical importance in vaccine development, as well as in ongoing quality control, as it enables candidate vaccines to be appropriately characterized and evaluated. In addition, the use of the WHO IS allows for the more-accurate comparison of the clinical performance of different candidate RSV vaccines. The WHO IS can there be used for the following:

1. To gain better understanding of acquired immunity to RSV through the standardized characterization of pre-existing and post-vaccination serum antibody responses to RSV in different patient populations, including those based on age (for example, infants, children or the elderly), geographical location or other (for example. pregnant women).
2. To assess RSV vaccine efficacy through comparison of the outcomes of vaccine trials when tested in different patient populations. This will allow regulators, developers and other interested parties to evaluate vaccine efficacy across different trials.
3. To assure the quality control of RSV vaccines as part of overall vaccine characterization and evaluation.

In line with the above, the WHO IS (NIBSC code 16/284) should be used when assaying neutralizing RSV antibody responses, and the results reported in IU along with information regarding the performance of the IS. This will allow the scientific community to fully benefit from the new standard and improve understanding of neutralizing antibody responses. Feedback from users will also help WHO and NIBSC to promote the use of the IS and to develop other standards and reagents that may further improve the standardization of assays used in the clinical evaluation of RSV vaccines. Detailed information on the IS can be found at: http://www.nibsc.org/products/brm_product_catalogue/detail_page.aspx?catid=16/284

In addition to the WHO IS, several BEI Resources RSV reference materials are also available (Table 2).

**Table 2**
**BEI Resources RSV reference materials**

| BEI catalogue number | Wyeth lot number | BEI lot number | Material | Format |
|---|---|---|---|---|
| NR-4020 | 06594 (called "Reference" in Yang et al. *(4)* | V327-501-572 | High-titre serum | 1 mL (lyophilized) |
| NR-4021 | 06937 (called "Control I" in Yang et al. *(4)* | V327-512-572 | Medium-titre serum | 1 mL (lyophilized) |
| NR-4022 | 06938 (called "Control II" in Yang et al. *(4)* | V327-513-572 | Medium-titre serum | 1 mL (lyophilized) |
| NR-4023 | | | Low-titre serum | 1 mL (lyophilized) |

| | | | | |
|---|---|---|---|---|
| NR-21973 | CBER RSV Ig Lot 1 | RSV-1 | Purified Ig | 2 mL (liquid) |
| NR-32832 | Testing panel | 63492188 | Panel contains:<br>• 1 vial NR-4020 (high-titre serum)<br>• 1 vial NR-4021 (medium-titre serum)<br>• 1 vial NR-4022 (medium-titre serum)<br>• 1 vial NR-21973 (CBER RSV Ig Lot 1)<br>• 1 vial: NR-49447 (Ig depleted serum) | Same respective formats as above |

BEI Resources reagents are shared with registered individuals and organizations conducting research on emerging infections and other relevant areas of interest related to microbiology. Registration with BEI Resources is required to request materials. Detailed information on the range of BEI RSV reference materials available, along with instructions on how to register with BEI Resources, are available at the BEI Resources website: https://www.beiresources.org/. Registered users may request reagents through the online BEI Resources catalogue. It should be noted that in order to ensure availability to all qualified researchers, BEI Resources policy is to provide only research quantities of a given reagent per year to each registered user. However, it is possible for over-the-limit requests to be met where there is appropriate justification. Such justification should be included in any request for an over-the-limit amount of any given reagent.

The BEI Resources RSV reference materials NR-4020, NR-4021, NR-4022, NR-4023 and NR-21973 were assessed for their ability to act as working standards in two multi-laboratory collaborative studies. Study results showed that all of these materials were able to reduce inter-laboratory variability in neutralization titres when used as standards. As large quantities of the BEI Resources RSV reference materials NR-4020 and NR-21973 are available, users may wish to consider using these materials as working standards or controls in their RSV neutralization assays.

Crank et al. *(5)* have reported the method shown in Box 1 for calibrating the BEI Resources RSV reference materials to the WHO RSV IS:

**Box 1**
**Calibrating BEI Resources RSV reference materials to the First WHO International Standard for antiserum to respiratory syncytial virus *(5)***

Neutralization was measured using a previously reported fluorescence plate reader neutralization assay with modification. Sera were diluted in threefold serial dilutions from 1:10 to 1:65 610, mixed with an equal volume of recombinant mKate-RSV expressing prototypic F genes from subtype A (strain A2) or subtype B (strain 18537), and incubated at 37 °C for 1 hour. Next, 50 µL of each serum dilution/virus mixture was added to HEp-2 cells that had been seeded at a density of 2.4 x $10^4$ in MEM (minimal essential medium) in each well of 384-well black optical bottom plates, and incubated for 23–24 hours before spectrophotometric analysis at 588 nm excitation and 635 nm emission (SpectraMax® M2e,

Molecular Devices, CA). The $IC_{50}$ for each sample was then calculated by curve fitting and non-linear regression using GraphPad Prism (GraphPad Software Inc., CA).

To standardize neutralization data to the WHO RSV IS (NIBSC code: 16/284), newly reconstituted IS was tested simultaneously with the BEI Resources RSV reference materials NR-4020, NR-4021, NR-4022, NR-4023 and NR-21973. Assays were performed with three different viral stocks, with each stock run on 3 different days by two operators to give a total of 18 runs. IU were assigned to each BEI Resources RSV reference material using the following equation as per the manufacturer's instructions:

$$\text{IU/mL} = \frac{\text{GMT BEI Resources standard}}{\text{(GMT WHO IS/2000)}}$$

GMT = geometric mean titre

The ratio of IU/GMT for the WHO IS in the assay was used to generate a conversion factor of 0.833. $IC_{50}$ readouts from RSV/A neutralization were multiplied by 0.833 to obtain IU/mL. The BEI Resources RSV reference material NR-4020 was included as a control in each neutralization assay run (Crank et al. *(5)* – Supplementary Materials p.4).

The following published method *(6)* provides an example of how the RSV neutralization titre of a serum sample might be determined, and how this neutralization titre can be converted to IU/mL.

## RSV neutralization assay method

**Aim**

To measure neutralizing antibody titres against RSV in serum samples

**Assay outline**

In this assay, serum samples are sequentially diluted and mixed with a fixed amount of RSV. The mixtures are then added to monolayers of HEp-2 cells and allowed to replicate for 24 hours. After this time-point, the cell layer is fixed and virus infectivity is detected via immunostaining. RSV plaques are detected by ELISPOT analysis and a 50% neutralizing titre is derived using CombiStats™.

**Reagents**

- PBS
- DMEM media
- DMEM, high glucose, without sodium pyruvate
- penicillin/streptomycin (Pen/Strep)
- amphotericin B (AmpB)
- L-glutamine 200 mM
- methanol
- bovine serum albumin (BSA)
- fetal calf serum
- 30% hydrogen peroxide ($H_2O_2$)
- biotynylated anti-RSV antibody

- ExtrAvidin®–Peroxidase
- Sigma*Fast*™ DAB substrate
- deionized water.

**Solutions**

- D10: DMEM + 10% fetal calf serum + 1% Pen/Strep + 1% L-glutamine + 1% AmpB;
- SF DMEM: DMEM + 1% Pen/Strep + 1% L-glutamine + 1% AmpB;
- D4: DMEM (without sodium pyruvate) + 4% fetal calf serum;
- Fixative: methanol + 2% $H_2O_2$ (1ml 30% $H_2O_2$ in 50 ml MeOH); and
- Staining diluent: 1% BSA in PBS.

**Cell preparation**

1. Seed HEp-2 cells in 96-well, flat-bottomed, cell culture plates at a density of 40 000 cells per well (400 000 cells per mL). Incubate the cells overnight at 37 °C; 5% $CO_2$. Cells are cultured and seeded in D10 medium.

**Neutralization assay**

1. Heat-inactivate serum samples by incubating in a water bath for 30 minutes at 56 °C.
2. In a sterile 96-well plate, dilute serum samples as shown below in SF DMEM:
   - make up at least 150 µL of 1:10 dilution in a sterile Eppendorf;
   - take 120 µL of 1:10 dilution and add to column 1 of duplicate sample rows;
   - add 60 µL of SF DMEM to columns 2 to 12 of sample rows;
   - perform 2-fold serial dilution (60 µL added to 60 µL) from column 1 to 12 and discard final 60µL from column 12;
   - add 60 µL of SF DMEM to Virus Only (VO) control wells; and
   - add 60 µL of SF DMEM to Media Only (MO) control wells.
3. Defrost virus stock as quickly as possible then dilute to 2x required concentration.
4. Add 60 µL of 2x required virus concentration to sample wells and VO control wells. Add 60 µL SF DMEM to MO control wells. Cover the plate and incubate for 1 hour at 4 °C.
5. Remove HEp-2 cells from incubator and wash gently with 200 µL of SF DMEM.
6. Add 100 µL of sample/virus mix to the cells and allow adsorption for 2 hours at 37 °C; 5% $CO_2$.
7. After adsorption, add 100 µL of D4 and incubate for 24 hours at 37 °C; 5% $CO_2$.

***Plate layout***

| | **1:10** | **1:20** | **1:40** | **1:80** | **1:160** | **1:320** | **1:640** | **1:1280** | **1:2560** | **1:5120** | **1:10240** | **1:20480** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Sample 1** | | | | | | | | | | | | |
| | | | | | | | | | | | | |
| **Sample 2** | | | | | | | | | | | | |
| | | | | | | | | | | | | |
| **Sample 3** | | | | | | | | | | | | |
| | | | | | | | | | | | | |
| **Virus only (VO)** | | | | | | | | | | | | |

| Media only (MO) |
|---|

**Immunostaining**

1. After incubation for 24 hours, remove medium from cells and gently wash once with 200 µL of PBS.
2. Fix the cells with 100 µL of fixative in a fridge for 20 minutes.
3. Remove fixative and wash gently with 200 µL of staining diluent (at this point, plates can be stored in the fridge in 1% BSA/0.1 % sodium azide/PBS until staining).
4. Prepare biotinylated anti-RSV antibody (dilution 1:500) in staining diluent and add 100 µL of the anti-RSV antibody solution to cells.
5. Incubate the cells with antibody for 2 hours at room temperature in the dark.
6. Remove the anti-RSV antibody solution and wash plates gently three times with 200 µL of staining diluent; gently blot the excess fluid onto tissue.
7. Prepare ExtrAvidin®–Peroxidase secondary antibody (dilution 1:500) in staining diluent and add 100 µL to each well.
8. Incubate the cells with antibody for 1 hour at room temperature in the dark.
9. Add 50 µL of Sigma*Fast*™ DAB substrate (prepared according to company instructions) to cells and develop in the dark.
10. Wash gently with 200 µL of deionized water to stop when fully developed and dry plates completely (a 37 °C dry oven may be used for quick drying).
11. Count plaques using ELISPOT reader.

**Calculating results**

Fig. 1 shows examples of virus-positive and virus-negative wells. The ELISPOT camera setting should be adjusted to obtain similar images, and the camera and count settings should be kept consistent for each laboratory.

**Fig. 1**
**Examples of virus-positive and virus-negative wells**

Positive well

Negative well

Exclude all wells without a complete monolayer from counting.

***Example plate count***

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sample 1 | A | 0 | 1 | 0 | 0 | 2 | 23 | 35 | 86 | 90 | 106 | 108 | 120 |
| | B | 0 | 0 | 0 | 0 | 6 | 35 | 51 | 68 | 100 | 112 | 136 | 138 |
| Sample 2 | C | 0 | 0 | 5 | 22 | 49 | 79 | 90 | 99 | 117 | 120 | 118 | 126 |
| | D | 0 | 0 | 6 | 15 | 40 | 66 | 96 | 112 | 125 | 134 | 125 | 143 |
| Sample 3 | E | 0 | 0 | 0 | 3 | 7 | 45 | 49 | 105 | 108 | 138 | 147 | 155 |
| | F | 0 | 0 | 0 | 3 | 5 | 40 | 55 | 99 | 111 | 133 | 144 | 152 |
| VO | G | 136 | 146 | 130 | 133 | 126 | 130 | 128 | 126 | 142 | 152 | 129 | 138 |
| MO | H | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Counts are then inserted into CombiStats™ and a 4-parameter logistic regression model used to calculate the 50% effective dose ($ED_{50}$). Further information on the use of CombiStats™ is available at the European Directorate for the Quality of Medicines & Healthcare (EDQM) website: https://www.edqm.eu/en/combistats

***Example graph and results table***


| Sample 1 | | | |
|---|---|---|---|
| Id. | Sample A | | |
| (ul/vial) | Lower limit | Estimate | Upper limit |
| Potency | 51.4056 | 63.4752 | 78.3611 |
| Rel. to Ass. | 51.4% | 63.5% | 78.4% |
| Rel. to Est. | 81.0% | 100.0% | 123.5% |
| ED50/vial | 865.541 | 1004.32 | 1165.60 |
| Rel. to Ass. | 865.5% | 1004.3% | 1165.6% |
| Rel. to Est. | 86.2% | 100.0% | 116.1% |

**Conversion of $ED_{50}$ titres to IU/mL**

Neutralization titre can be converted to IU/mL using the following formula (1 mL of the WHO RSV IS contains 2000 IU):

$$\text{IU/mL} = \frac{\text{GMT sample}}{(\text{GMT WHO IS}/2000)}$$

GMT = geometric mean titre

***Example***

Sample titre = 1200
WHO IS titre ($ED_{50}$) = 1500
Calculation = 1200/(1500/2000) = 1200/0.75 = 1600
Neutralization titre of sample = 1600 IU/mL

**References**

1. McDonald JU, Rigsby P, Dougall T, Engelhardt OG and study participants. Establishment of the first WHO International Standard for antiserum to respiratory

syncytial virus: report of an international collaborative study. Vaccine. 2018;36(50):7641–9 (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6838659/?msclkid=fced8cf1c56711ec971002a15e19aba8, accessed 26 April 2022).

2. McDonald JU, Rigsby P, Atkinson E, Engelhardt OG and study participants. Expansion of the 1st WHO international standard for antiserum to respiratory syncytial virus to include neutralisation titres against RSV subtype B: an international collaborative study. Vaccine. 2020;38(4):800–7 (https://www.sciencedirect.com/science/article/pii/S0264410X19314951?via%3Dihub, accessed 26 April 2022).

3. Knezevic, I. 1st WHO International Standard antiserum to RSV: availability and benefits for RSV vaccine development. Commentary on the collaborative study report published in the vaccine journal (Vaccine 2018;36:7641–9). Vaccine. 2019;37(31):4233–5 (preview: https://www.sciencedirect.com/science/article/pii/S0264410X19307832?via%3Dihub, accessed 26 April 2022).

4. Yang DP, Zielinska E, Quiroz J, Madore D, Rappaport R. Preparation of a respiratory syncytial virus human reference serum for use in the quantitation of neutralization antibody. Biologicals. 2007;35(3):183–7 (abstract: https://pubmed.ncbi.nlm.nih.gov/17241789/, accessed 26 April 2022).

5. Crank MC, Ruckwardt TJ, Chen M, Morabito KM, Phung E, Costner PJ et al. A proof of concept for structure-based vaccine design targeting RSV in humans. Science. 2019;365(6452):505–9 (abstract: https://www.science.org/doi/10.1126/science.aav9033, accessed 26 April 2022).

6. Jorquera PA, Anderson L, Tripp RA. Human respiratory syncytial virus: an introduction. Methods Mol Biol. 2016;1442:1–12 (abstract: https://pubmed.ncbi.nlm.nih.gov/27464683/, accessed 26 April 2022).