WHO manual for the establishment of national and other secondary standards for antibodies against infectious agents focusing on SARS-CoV2

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The first draft of this manual was prepared by a drafting group comprising: Dr Ian Feavers, Consultant, UK; Dr Micha Nuebling, Paul-Ehrlich-Institut, Germany; Dr Peter Rigsby, Dr Giada Mattiuzzo and Dr Mark Page, National Institute for Biological Standards and Control, UK; Dr Ligia Pinto and Dr Troy Kemp, National Institutes of Health, USA; Dr Gagandeep Kang, CMC Vallore, India; Dr Youchun Wang, National Institutes for Food and Drug Control, China; and Dr Ivana Knezevic and Dr Dianliang Lei, World Health Organization, Switzerland.

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Glossary

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 specific antibodies against an infectious agent.

Baseline parameters. The optimal storage conditions that retain biological or immunological activity and are used for comparative purposes.

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 (1).

Calibrator. A material used to adjust the output from a manufacturer's product based on or traceable to a reference preparation.

Commutability. The property of a reference material, demonstrated by the equivalence of the mathematical relationships among the results of different measurement procedures for a reference material and for representative samples of the type intended to be measured (2).

Functional antibody assay. A bioassay that measures the immunological activity of an antibody that reduces disease (e.g. neutralising, opsonophagocytic or complement-mediated activity).

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

International biological measurement standard. Commonly referred to as WHO International Standard (IS). A biological substance, i.e. one that cannot be fully characterized by physico-chemical means alone and is measured using a bioassay, that 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 Standard.

Measurand. The object being measured in an assay.

Neutralising antibodies. Antibodies that render a virus or toxin ineffective.

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

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

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) standards. Reference standards that are calibrated against and traceable to WHO IS established by regional or national authorities, or by other laboratories.

Tertiary (reference) standards. Reference materials, such as working reagents or standards, product calibrators or control materials, calibrated against the secondary standard.

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 having stated uncertainties.

Uncertainty. An estimate attached to a test result which 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 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.

Introduction

The development and implementation of international reference standards for biological materials is a core function of the WHO that has an important impact on the high quality and consistent dosing of biological medicines used worldwide. These standards are widely used in the development, evaluation, standardization and control of products in industry, by regulatory authorities, as well as in biological research in other scientific organizations.

WHO International Standards (ISs) are established by its Expert Committee on Biological Standardization (ECBS) with an assigned International Unit (IU). Metrologically ISs serve as the primary standard for calibration of national and other secondary standards and are considered to be of the highest order. Consequently, it is important to conserve the limited stocks of an IS and to this end national authorities frequently consider the establishment of their own secondary reference materials. 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 preparations should be calibrated in IU by direct comparison with the respective IS.

The ECBS developed recommendations for the preparation, characterization and establishment of international and other biological reference standards in 1978. This document was last revised in 2004 and is available in the WHO Technical Report Series 932 https://www.who.int/publications/m/item/annex2-trs932 (3). Subsequent feedback from National Control Laboratories (NCLs), vaccine manufacturers and diagnostics producers led to the publication of manuals to address practical issues in the establishment of national and secondary standards for vaccines https://www.who.int/immunization/documents/who_ivb_11.03/en/ (4) and in vitro diagnostic assays for infectious diseases based on nucleic acid or antigen detection https://www.who.int/biologicals/WHO_TRS_1004_web.pdf (5).

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), the aetiological agent of COVID-19, causes mild or asymptomatic infection in the majority of cases; however, about 10% of cases require medical intervention and a small proportion result in severe pneumonia and death. The COVID-19 pandemic has led to an major global effort to develop vaccines and therapeutics, including antibody-based therapeutics. In December 2020, ECBS established the first WHO IS for anti-SARS-CoV-2 immunoglobulin to facilitate the development and harmonisation of serological assays to a common unitage (6). These assays provide information for establishing correlates of protection and are essential to support the clinical development of vaccines and therapeutics. They also have the potential to support serological diagnosis of infection or confirmation of vaccination status, and the seroepidemiological studies required to assess the impact of COVID-19. They broadly fall into two categories: virus neutralisation assays and antibody binding assays such as ELISAs. Plaque or foci reduction neutralisation tests (FRNT or PRNTs) are widely regarded as the gold standard for measuring potentially protective antibodies against many viral diseases. These assays involve the use of live virus, which in the case of SARS-CoV-2 requires laboratories at containment level 3; the use of pseudotyped virus in neutralisation assays has been shown to be a suitable alternative (7).

Respiratory syncitial virus (RSV) is a significant cause of lower respiratory illness in infants, the elderly and immunocompromised, and the development of a vaccine remains a global priority. Activity in this area has increased in recent years and in 2017 ECBS recommended

the establishment of the First WHO IS for antiserum to RSV. This was initially recommended for use in the assessment of RSV subtype A neutralization titres in human serum but was extended to include subtype B in 2019.

Current HPV vaccines are based on virus-like particles consisting of recombinant capsid proteins. The standardisation of assays for HPV capsid antibody has supported vaccine developments and continues to underpin epidemiological studies. In recent years WHO ISs have been established for virus serotypes 16 and 18.

The worldwide demand for the anti-SARS-CoV-2 and many other antibody standards (e.g. RSV and HPV) has inevitably led to the development of national and other secondary reference materials. Thus, in addition to manuals for secondary standards for vaccines and in vitro diagnostics that rely on DNA or antigenic components for virus detection, the increasing demand for antibody standards has highlighted the need for a manual addressing the calibration of secondary standards for the evaluation of antibody responses to infection and vaccination.

Use of 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 (1), which in the physical sciences means obtaining values in SI units. Critically, 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 ECBS to measuring biological activity is to establish the highest order reference material, 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, it is critical 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 ISs, 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 as an in-house standard, a run control, a working standard or a calibrator in manufacturers' products.

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

Table. Key properties of WHO IS, secondary standards and tertiary standards (from TRS 1004)

Property	WHO IS	Secondary standard	Tertiary standard
Alternative names	Highest order, international conventional calibrator	Regional or national reference materials, laboratory or manufacturer's working calibrator	Working reagents or standards, manufacturer's product calibrator, control material
Calibration	Evaluated in an international collaborative study, involving laboratories worldwide, different assays and different types of test 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)
Communtability	Must be determined experimentally relative to clinical specimens	Should be determined experimentally relative to clinical specimen	Consideration should be given to experimentally determining relative to clinical specimen
Material	Should resemble, as closely and as feasibly as possible, the analyte being measured – for example, for assays for viral nucleic acids the standard will be the wild-type patient-derived virus in plasma (the normal sample type analysed)	Should resemble, as closely as possible, the analyte to be measured. However, for assay-specific secondary standards, synthetic materials such as armored RNA, plasmids and recombinant proteins, may be used and laboratories are encouraged to address commutability	Should resemble, as closely as possible, the analyte to be measured. Biological material similar to the tested sample, or nonbiological materials, such as armored RNA, plasmids and recombinant proteins may be used, and laboratories are encouraged to address commutability
Typical final format of standard	Lyophilized	Lyophilized or liquid	Liquid
Usage	Calibration of secondary standards; initial validation of new assay/platform	Calibration of tertiary standards; working standards; run control; calibrator	Working standards; run control; calibrator
Establishment of standard	International agreement through a WHO international collaborative study, proposal for adoption and subsequent establishment by the WHO Expert Committee on Biological Standardization	May be calibrated in several ways: 1. In parallel with a study to establish the IS. 2. Regional or national collaborative study similar to the WHO collaborative study but with fewer participants. 3. Small study by one or a limited number of laboratories with a single assay or a limited number of different assays/ platforms.	1. Assay-specific study, normally by a single laboratory for use with a specific test/platform. 2. Small study by a limited number of laboratories with a single assay or a limited number of different assays/ platforms

Often the IS has yet to be established at the time of early clinical studies and antibody assays are standardised using an antiserum working reagent. This is particularly likely to occur in emergencies (e.g. the Covid-19 pandemic) when vaccine and therapeutic antibody development proceeds at pace before sufficient convalescent serum is available to produce the IS. As long as sufficient working reagent is retained, it can be used to convert the results of these early studies into IU retrospectively, once the IS has been established.

Scope of document

Antibody reference standards are used to ensure uniformity in the designation of potency or activity to immune sera and antibody preparations, and minimise systematic deviation of assays. Although they can be used to qualify or validate in vitro diagnostics and other test procedures, the scope of this document is limited to the calibration secondary standards for use in evaluating antibody responses elicited by natural infection or vaccination. Such standards may also be used to ensure consistent dosing of convalescent plasma and monoclonal antibodies in the treatment of infection. The qualification or validation of serological test procedures is typically achieved using panels of low, medium and high titre sera calibrated against the IS and is beyond the scope of this document.

The term secondary standard is used to include all standards developed by regional or national authorities, or by other organisations and calibrated against the IS. Such secondary standards are designed to provide greater quantities of calibrated material, for which the IS is not available due to limited supply.

Although this document is primarily aimed at the development and calibration of secondary standards for the evaluation of antibody responses to SARS-CoV2, it is not limited to SARS-CoV2 and many of the principles are derived from the development of antibody standards for other infectious diseases. Therefore, this manual is suitable for laboratories wishing to establish secondary standards for the evaluation of antibody responses to any infection.

The principal serological methods used to evaluate antibody responses to SARS-CoV-2 are neutralisation assays and antibody binding assays. The former include assays that use either live or pseudotyped viruses. The latter include various ELISAs and flow cytometry assays, some of which use the viral spike protein as the target antigen but many use other antigens and often consist of a multiplex format. Reagents such as the viruses and pseudotyped viruses used in neutralisation assays, and antigens used in antibody binding assays are not covered by this manual as by definition they are not standards.

This document provides general guidance on the principles of the preparation of secondary standards. Specific issues associated with the preparation of any particular standards must be considered on a case-by-case basis.

Principles for preparing secondary standard for antibodies

Compared to other biological reference preparations, a polyclonal antibody standard for a defined infectious agent is unique since a polyclonal serum or plasma contains different analytes (measurands) of different potencies, with each of the analytes defined by both the antigen / epitope against which it is directed, and its antibody class (e.g. IgG, IgA, IgM). Assays to be harmonised by an antibody standard are either measuring a biological activity, e.g. neutralization capacity, exhibited by subsets of antibodies, or are measuring binding antibodies, characterized by the target antigen(s) and the antibody class(es) detected, dependant on the assay design. Each analyte (measurand) present in the standard and of interest for harmonisation of respective assays must be formally defined by unit and quantity.

Antibody standards 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 immunological characteristics of the standard should be comparable with the samples to be tested.
- They have a formally defined unit and quantity, assigned using appropriately designed studies and assays.
- They are stable with respect to that formally defined unit and quantity.

Procedures for the production of ISs are detailed in WHO Recommendations for the Preparation, Characterization and Establishment of International and Other Biological Reference Standards (3). In general, these high-level requirements are equally applicable to secondary standards but with some critical differences that may allow 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 measurand (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 about a single underlying true value or a probability distribution of values to estimate uncertainty. In such cases, uncertainty cannot be attached to the reference material but may be assay-specific.

- Value-assignment methodology - The traceability path and uncertainty are only valid for the assay methodology used to assign the value of standard. For some ISs, units are assigned for specific assays (e.g. in virus neutralisation 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 re-calibrate 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 - Stability of a secondary standard is usually monitored in real-time against the IS. Ideally this should be checked by on-going monitoring of a suitable parameter appropriate for the assay used (e.g. NT_{50}).
- Commutability – Commutability is the extent to which the reference standard is suitable as a standard for the various samples being evaluated. When appropriate and feasible commutability should be assessed as part of a collaborative study by including a panel of different samples for which the standard will typically be used.

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

This document explains in detail issues that must be considered in the preparation of secondary antibody standards. ISs are likely to be lyophilised 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 it does not interfere with the assay, affect its stability or safety, and 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, countries are recommended to collaborate in the development of regional standards whenever possible to minimise duplication of effort.

Planning

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 three to five 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 and it may be informative to survey likely users of the secondary standard.

- 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 neutralisation or opsonisation, and 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 a single or multiple antibody specificities?
- What is the ideal shelf life of the proposed secondary standard?
- Is the material infectious and what precautions can be taken to mitigate any risk to users?

The likely annual demand can therefore be determined, 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. Records to be kept at each stage of the project are detailed in Appendix 1.

Selection of candidate material

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

The pool may consist of plasma or serum from convalescent or vaccinated individuals depending on the intended application of the standard. The specificity of the antibodies in the standard will depend on the source of material and therefore needs careful consideration when planning the project. In general, convalescent plasma or serum will have a broader antibody specificity against an infectious agent than plasma or serum from vaccinees, which will contain antibodies specific for the vaccine antigens. For example, many of the SARS-CoV-2 vaccines are based on the S antigen and therefore anti-S antibodies will be predominant in plasma from these vaccinees.

To ensure the safety of the standard, individual donations should be negative for known blood-borne virus markers (e.g. HIV and hepatitis viruses) and, if necessary, the treatment of candidate material by an appropriate validated method to reduce the risk of virus contamination should be considered. For example, the risk of the presence of enveloped viruses may be reduced by 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 clear to all parties during handling and transport. An MTA can also be used to ensure all parties adhere to specific legal and ethical considerations relating to the material.

Sufficient volume of bulk material should be filled so that the standard will last for three to five years (see planning above). Although relatively large volumes of plasma may be obtained from healthy adult volunteers (e.g. by plasmapheresis), this typically means that plasma donations from a number of individuals will be required. Sufficient time should be allowed between the onset of symptoms or vaccination for the antibody response to be induced. Individual donations should be characterised by a laboratory experienced in the immunoassays that will use the standard and based on the resulting data a decision can be made on which donations to include in the final pool.

The pooling procedure should ensure that the material is mixed thoroughly and is homogeneous. Care should be taken to avoid denaturation of protein during mixing. In addition to any studies on the individual batches before pooling, the homogeneous blend should also be characterised to demonstrate its suitability 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 minimised. For example, samples can be taken from plasma donations for characterisation 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 the immunological properties of the material are conserved.

Processing of final container

Quality aspects

Although manufacturing of reference standards does not require adherence to 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 and process times and cycles) should be documented and any equipment used for manufacture or QC testing must be kept in recordable certification. Once QC testing is available, the manufacturing process and product attributes should be reviewed and approved before the standard is distributed.

Nature of the secondary antibody standard

Antibody standards may be lyophilized, liquid or frozen liquid. They are generally lyophilized, as experience has shown this is a consistently stable format that facilitates distribution. Although this is the preferred option, there may be circumstances in which immunological characteristics of the standard are affected by lyophilisation or subsequent reconstitution of the material. If lyophilization is not possible or desirable, 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 thawing of frozen standards should be avoided because of its 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 freeze thaw does not affect the activity of the material.

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 elasotomeric closures, such as rubber stoppers may have inferior storage stability to those supplied in flame sealed glass ampoules, the preferred container for ISs. 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 should be assessed for the chosen storage conditions, 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 (e.g. to meet biocontainment requirements), in which case they should also be of pharmaceutical quality.

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 (e.g. virus neutralisation or opsonophagocytosis) or where an assay requires the subsequent culture of the infectious agent (e.g. complement-mediated killing). Although strict sterility is not always required and may not be easily achieved in practice, it is

advisable to minimise 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 minimise the contamination of the material during filling and drying where applied. Suitable environmental monitoring, including particle and microbial monitoring of the process area and also appropriate batch testing of the candidate standard will be required.

Accuracy/consistency of fill

The filling process should be well-controlled so that the dosage of active reference standard is within tightly defined limits and consistent across the batch. Although this limit may not need to be as tight for secondary standards, it should 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 it is used. For information, ISs are typically filled within a coefficient of variation of 0.25%. 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.

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 product temperature 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 product with low residual moisture. At the end of drying the product should be stoppered in either a vacuum or a dry gas environment that will prevent any atmospheric moisture ingress into the container on storage. Antibody standards are typically stored under a dry, inert atmosphere such a nitrogen.

For information, the freeze-drying cycle for the first WHO IS for SARS-CoV-2 immunoglobulin was as follows: 1) material was frozen at -50°C for 4 hours; 2) primary drying was at -35°C for 40 hours at 100μbar vacuum; 3) temperature was ramped to 25°C over 10 hours; 4) secondary drying was at 25°C and 30μbar vacuum; 5) vials were back filled with dry nitrogen at atmospheric pressure. An example of an SOP for filling an IS can be found in Appendix 2.

Characterisation

Before a candidate secondary standard is calibrated against the IS, its identity should be confirmed using a suitable assay to demonstrate it has the expected immunological activity.

Other tests which should be performed on the candidate standard are:

Appearance
A freeze-dried standard should have a consistent, well-formed cake. Collapsed freeze-dried material is often associated with high residual moisture and poor stability. Inconsistencies amongst individual containers should be investigated. The appearance of the reconstituted product should be checked for consistent appearance and absence of particulate matter. Liquid and frozen standards should also be examined for their appearance and lack 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%, although higher levels of residual moisture may be acceptable for secondary standards providing 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 (e.g. EP or USP) guidelines. Other assays should be validated or qualified as appropriate.

Baseline parameters, such as moisture content and potency, may be set at this time to evaluate the stability of the product in the future.

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 blood-borne infectious agents using validated procedures and this may be reaffirmed by testing material in the final container.

Calibration against the International Standard

Principles of calibration

Calibration is the process by which a concentration is assigned to a reference by the direct comparison of measurements with a higher order reference, and represents one of the crucial stages of 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. This guideline describes the minimum requirements for the calibration of secondary standards intended for use in a specific method in one laboratory (single assay calibration) and by more than one laboratory in multiple methods (i.e. a collaborative study 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.

Collaborative study design

The purpose of secondary standards is for the harmonisation of assays measuring defined analytes contained in the IS (biological activity, binding antibodies of different type of immunoglobulins and specificities). Therefore, only assays measuring the same analyte (measurand) are included in the respective assessment of data. Harmonisation of assays and commutability of the reference preparation is investigated by inclusion of a set of various routine clinical samples, e.g. representing different stages of infection, different infection courses, different antibody titres and antibody classes. However, in a single collaborative study, assays of different design and measuring different analytes may be included, providing that subsequent data analysis carefully differentiates between the individual analytes. A candidate secondary standard is estimated fit for purpose only if both its capacity for harmonisation of specific assays and its commutability are proved by the collaborative study results.

Secondary antibody standards used by multiple laboratories (e.g. different manufacturers and NCLs) should be calibrated directly against the current IS in a collaborative study. Ideally, the collaborative study should be organized 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 the selection of study participants. The calibration study should follow sound statistical principles (see below). Due to the complexity of the reported data, which typically includes data from different types of assays, the statistical analysis should be performed by a statistician. The general principles of planning and executing these types of collaborative study are described in Chapter 6 of the WHO manual for the preparation of biological reference standards (3).

The number of 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, the availability of suitably experienced participants and their resources. For a secondary standard, the number and geographical origin of the 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 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 participants but, in principle, there should be sufficient participants to generate an adequate number of data sets when assays are variable. Where there are few participants, a larger number of independent assays 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 a standard assay performed by all participants and the SOP should be provided by the WHO collaborating centre in advance.

Prior to the start of the study, information on the assays the participants are proposing to use should be obtained. A study protocol should be sent to all participants along with a form to be returned with information on and the results of each assay. Specimen templates for the collaborative study documentation are included in appendix 3.

Single assay calibration

In some instances, the calibration may be carried out by a single laboratory with experience of the relevant assay(s), e.g. a vaccine manufacturer or local NCL. In such circumstances, a larger number of independent assays may have to be performed, to ensure sufficient precision of the assigned potency (see below).

Statistical analysis

This guideline reflects the common statistical methods used for the calibration of reference materials. Any other statistical method, which has been demonstrated to be a reliable approach to calibrate such materials, may also 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.

Statistical models

The calibration study data should be analysed using the relevant statistical model for the assay, for example, using the methods recommended by the WHO or European Pharmacopoeia. The statistical validity of the fitted model should be assessed for each individual assay. For the parallel-line and probit models, as the most appropriate and proven statistical methods for this analysis, the linearity and parallelism of the logarithmic dose-response relationships between the IS and secondary standard should be evaluated. If the assay response is linear and the response lines are parallel the estimate of the relative potency of the candidate secondary standard against the IS can be calculated. Using the parallel line model validity criteria of the linearity could be the coefficient of determination (r^2) or a significance test for non-linearity (8). Parallelism could be demonstrated by means of a significance test for non-parallelism or an equivalence approach for the difference or ratio of slopes (i.e. the confidence interval for the ratio of slopes must entirely lie in between pre-defined equivalence margins). In addition, the precision with which the 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. 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 per milliliter (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 (3). The variability of the vial weight during filling for each IS is quoted in the study report and the Instructions for Use accompanying the standard.

Single assay calibration

The IS and the candidate secondary standard should be tested in a minimum of three independent assays. The candidate material should be tested neat (where possible) and at a minimum of two further (e.g. two-fold) dilutions within the linear range of 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 in duplicate. The results obtained with the parallel line analysis (if necessary on log transformed data) should be used to give a “relative potency” of the secondary standard against the IS in IU/mL. The parallel line or curve analysis should be the preferred option for data analysis.

Collaborative study calibration using multiple assays

Results from all participants should be analysed by statistical methods described and considered appropriate by the responsible statistician. This analysis typically requires access to suitable computing facilities and statistical software. The testing requirements and protocol of each laboratory/test should follow the protocol described for the single assay calibration. The results of each assay method should be analysed separately and should provide an estimate of the relative potency and precision of the candidate secondary standard against the IS.

The variation in results between test methods, and between laboratories, should be described and assessed as part of the statistical analysis (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 the statistical point of view, exclusion of data should be taken into account in subsequent analysis where striking differences of results within assays, between assays, between participants or test methods are observed. All valid potency estimates for the candidate secondary standard should be combined to produce a final geometric mean potency/content with 95% confidence limits. It is useful to display and assess the results graphically, e.g. as histograms or scatter plots.

Calculation of uncertainty of measurement

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 measurement of uncertainty is a complex area and where possible advice should be sought from a statistician.

The uncertainty, often referred to as measurement uncertainty (MU) expresses the 95% confidence limits either side of the observed value assigned to a product. By estimating the MU of a product the confidence in the final value assigned is shown. Uncertainty should be calculated using log transformed data. Where no MU is assigned a justification for this should be provided e.g. when the calculated uncertainty is negligible in comparison to the 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 uncertainty (9,10). A typical approach to estimate 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 (i.e. under the exact same conditions) and combine the results from at least three independent tests. The more times the sample is tested the better, in order to reduce the magnitude of measurement uncertainty. In calibration for a single assay, the test system used should be of the highest order possible, i.e. 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 (8) 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.

Stability

Understanding the stability of reference standards is important to: estimate its shelf-life in storage for its intended use; identify appropriate conditions for distribution to users; and determine its shelf-life following reconstitution. Continued evidence of stability can be acquired from the experience reported by users and monitoring the long-term stability, in real time, against the assigned potency of the IS. The application of predictive models of stability (e.g. the Arrhenius model), which are used during the development of ISs where there is no higher order reference material, are not generally necessary with secondary standards whose stability can be assessed by reference to the IS. In general, the antibody activity of freeze-dried antisera and plasma are stable at -20°C. Where this is not the case, the stability of frozen or liquid preparations should be determined experimentally. Once reconstituted, diluted or aliquoted, users should determine the stability of the material according to their own method of preparation, storage and use.

The reference standards should be granted official status for use on the basis of existing data including long term stability data generated on the material, the consistency of the data generated in the assay and the regular assessment done 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.

Collaborative study report

An outline of the collaborative study report indicating required information is given in Appendix 3b.

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 (e.g. using an automated temperature monitoring system), and alternative storage arrangements should be available in case of breakdown. The use of frost-free freezers is not recommended as the temperature cycles vary more widely than for 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 from the use of the standard from as many users as possible (e.g. neutralising antibody or antibody binding titres). Where the data indicate a possible stability issue, further investigations should be undertaken, such as a small collaborative study amongst laboratories familiar with the use of the standard.

The stability of the standard should be assessed periodically relative to the IS. The frequency will be dependent on the precision of assays and the predicted stability.

Responsibilities of the custodian laboratory

The custodian laboratory is responsible for the following:

- Storage of the secondary standard under appropriate conditions
- Distribution of secondary standard when requested under appropriate conditions
- Maintenance of complete records on project
    - The source of the bulk standard and its characterisation, 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
- Maintain awareness of relevant assay developments and how the standard is being used
- Monitor stability by requesting feedback on the use of the secondary standard that might contribute on-going evidence of the stability of the material
- Publish the results of the collaborative study
- Provide advice and training on the use of the standard

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

Instructions for use and labelling

All ampoules or vials of the secondary standard should be labelled with the name of the custodian institution, the name of the material, any assigned code number, the assigned potency, the storage temperature and that the material is ‘Not for use in humans’.

Each package of secondary standard should include a data sheet/instructions for use, which should contain the following information:

- the storage and shipping conditions
- the potency of the standard
- the type of 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 conditions of transport
- relevant safety information
- available information about stability must be clearly specified and 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 on the label
- information on stability of the standard once reconstituted, diluted or aliquoted
- contact information for feedback on any issues relating to the use, quality or stability of the standard
- appropriate safety data
- reference to the collaborative study report

Dispatch of standards

Standards should be dispatched under appropriate conditions for 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 to avoid multiple freeze thaw cycles unless stability studies have shown this to be unnecessary. Instructions for use 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 material. This should take into account any residual risk that the material is infectious and be documented for each standard.

Batch replacement

Replacement of a secondary standard needs to be planned and timely. The process as described above should be followed, including calibration of the replacement against the IS and not the previous secondary standard. Although the previous standard may be included in the study, it should not be used for calibration of the replacement material as this increases the risk of the assigned value drifting. Only if an IS is not available should the calibration be made against the previous batch of secondary standard.

If surplus plasma or sera pool is available that has been stored under appropriate monitored conditions, this excess could be used to allow replacement with identical material.

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

References

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3) 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 Technical Report Series 932; 2006: Annex 2

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6) 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. 66–68; https://www.who.int/publications/i/item/9789240024373).

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9) Bell S. A beginner's guide to uncertainty of measurement. Measurement good practice guide No. 11. (Issue 2 with amendments 2001). National Physics Laboratory, Teddington, Middlesex, UK, 1999.

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Appendices

Appendix 1: Documentation to be compiled during a standardisation project

1) Information on plasma pool e.g. source of individual donations, characterisation of donations, ethical approval and other relevant correspondence.

2) Characterization technical records (fill logs/details)

3) Collaborative study raw data

4) Collaborative study reports

5) Documentation recording the decision-to-establish by appropriate authority

6) In-use scientific feedback (including stability)

Appendix 3: Collaborative study documentation

3a: Specimen templates for Invitation/Questionnaire, Study Protocol Results form

Specimen invitation to participate in a collaborative study

Dear……..

I am writing on behalf of xx to invite you to participate in a collaborative study to establish a national/regional Standard for xx. The aims and provisional structure and timelines of the study are set out in the attached draft study protocol. The study will involve testing the IS and x candidate antibody standards in xx many assays.

May we ask you to:

1) Confirm if you are able to participate in this study

2) If you are, fill in the attached methodology questionnaire

3) Offer any comments on the proposed study protocol relevant to your contribution.

It is normal practice to acknowledge 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 also in subsequent publications.

Thank you for considering this request. We hope you can agree to participate.

Yours sincerely,

######################

Specimen Questionnaire

Name of participant:
Address:
Telephone
Email

I would like to*/am unable to* participate in the Collaborative Study to assess the suitability of candidate reference material to serve as the National Standard xxxxx.
(* delete as appropriate)

Include any additional information required for shipping materials

Brief description of method

Antibody assays routinely performed

Signed:

NAME

Date:

Please return to:
xxxxxxxxxxxxxx
Email address xxxxxxxxxx

Specimen Draft Protocol for a collaborative study to assess the suitability of candidate secondary reference material for xxx

Background

Including need for standard, availability+ information on IS, specifications

Information on materials to be included in the study

IS

Candidate Standard

Any other samples

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

Assay Methods

WHO/pharmacopoeial and/or methods in use in laboratory

Design of study

Number of assays

If more than 2 samples (IS + candidate secondary standard) are being tested, emphasize inclusion of all study samples in each assay.

Results and data analysis

Supply data sheet so that all essential information can be recorded.

A separate data sheet should be completed for each experiment.

Timelines

Include deadlines for return of results

Result sheet – 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, responses (OD, plaque number etc), data from relevant controls

Return to xxx;

Email address

3b: Collaborative study report - Outline of the contents required

Introduction

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

Materials

- Candidate standard – description of source of plasma, including ethical considerations; whether individual donors were convalescent or vaccinated; how donations were treated; characterisation of individual donations; describe how they were pooled and the rationale for excluding particular donations; any treatments applied to the bulk pool (e.g. defibrination); the identifying code of the candidate reference standard.
- Other study samples
- IS name and code

Participants

List participants and their locations

Study design and Assay methods

Set out study design and refer to the study protocol
Indicate suggested dilutions for materials
Provide plate template
Include number of assays participants requested to perform
Describe stability study

Results

Include statistical analysis, identity blinded if appropriate

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

Discussions/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 analysis generated by calibrations 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 packages that have been widely used in the calibration of biological standards:

1) WHO Bioassay Assist

Bioassay Assist is a statistic analysis software for the quality control of biological products donated to WHO by the National Institute of Infectious Diseases, Japan, for users agreed by WHO. This software is consists of calculation and data analysis functions, including parallel line and Probit assays, the two methods most frequently used in bioassays.

This software is provided freely upon request. To request this software, please contact Dr Dianliang Lei (leid@who.int), Norms and Standards for Biologicals, Technical Specification and Standards Unit, Health Product Policy and Standards Department, Access to Medicine and Health Products Division, World Health Organization, Avenue Appia 20, CH-1211 Geneva 27, Switzerland.

2) CombiStats:

This package is intended for the statistical analysis of data from biological dilution assays or potency assays. It includes the following models: parallel line, slope ratio, Probit, 4- and 5-parameter logistic curve, and single dose models as well as ED50 calculations.

This software is available EDQM, Council of Europe, on payment of a license. For further information see www.edqm.eu/en/combistats

Other useful software include:
ELISA for Windows (Plikaytis, B.D, Carlone, G.M., Program ELISA *for Windows* User's Manual, version 2. Centers for Disease Control and Prevention, Atlanta, GA, U.S.A., 2005). Available from CDC at https://www.cdc.gov/ncird/software/elisa/index.html

The IU ELISA calculator provided by Dr. Dillner, Karolinska Institute, Sweden at http://188.114.242.3:8080/IUWeb/

Appendix 5: SOP of ELISA for SARS-CoV-2 antibodies.

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. Firstly, antigen is coated onto a microplate, then plasma/sera samples are added. Any bound antibody from these is then detected by an appropriate anti-species peroxidase-conjugated antibody. The antibody complex is then detected with a TMB substrate.

HEALTH AND SAFETY

The health and safety aspects of this work are governed by: Institute Safety Compendium Code of Practice for laboratories 3044 (s/n 5403) and 3045 (s/n 5464).
Wear suitable personal protective equipment as stipulated in the relevant risk assessment (lab coat, nitrile gloves, eye protection). Avoid contamination of equipment and surfaces.

EQUIPMENT AND MATERIALS

- Flat-Bottom NUNC maxisorp 96-Well Plates (Fisher Scientific #44-2404-21, or equivalent)
- Phosphate Buffered Saline (1X) (Gibco #10010-023, or equivalent)
- Tween 20 (Fisher Bioreagents #BP337-500, or equivalent)
- Milk Powder (Marvel, or equivalent)
- TMB Substrate (Neogen #309175, or equivalent)
- 1N Sulphuric acid H_2SO_4 (Sigma-Aldrich #**339741,** or equivalent)
- Polypropylene sterile conical tubes: 15 mL 50mL
- Sterile, serological pipettes: 5mL, 10mL, 25mL
- Micropipette tips: 10µL, 20 µL, 200 µL, 200 µL, 1000 µL
- Sterile reservoirs (Fisher Scientific #07-200-127, or equivalent)
- Multichannel pipette(s): 200 µL
- Wypalls
- Pipet-boy
- Class II biological safety cabinet
- Ultra-Low Freezer (-80°C)
- Refrigerator at 4°C (+/- 1°C)
- Wellwash versa ELISA plate washer (Thermo Scientific) or equivalent
- Fluostar Omega microplate reader (BMG) or equivalent

Proteins

Several sources of proteins are available
NIBSC/CFAR (kindly donated by Dr Peter Cherepanov, The Francis Crick Institute, London, UK)
SARS-CoV-2 S1 (#100979)
SARS-CoV-2 RBD (#100981)
SARS-CoV-2 N (#100982)

Other sources are Native Antigen company, we have tested successfully SARS-CoV-2 S1 and S2 (https://thenativeantigencompany.com/coronavirus-dashboard/)

For stabilised trimeric spike, plasmids are available from Dr Barney Graham (NIH/NIAID, Bethesda, MD,USA) and Dr Florian Krammer (Icahn School of Medicine at Mount Sinai, New York, NY,USA) for research use

Antibodies

- Secondary antibody: Anti-Human IgG (Fab specific)-Peroxidase antibody produced in goat (Sigma #A0293) (use at 1 in 3000)
- Positive control for anti-S1/RBD/Spike: Anti-COVID-19 & SARS-CoV S glycoprotein [CR3022], Human IgG1, Kappa (Absolute antibody, Ab1680.10) Positive control - dilute to 0.5µg/ml
- Research reagent, human convalescent plasma from COVID19 patient: https://nibsc.org/products/brm_product_catalogue/detail_page.aspx?catid=20/130
- Positive control for Nucleoprotein: SARS-CoV-2 (2019-nCoV) Nucleoprotein / NP Antibody, Rabbit Mab (Sino Biological, 1018140143-R019-SIB) – dilute to 0.5µg/ml
- Related secondary antibpdy: 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 50µl of antigen at 1µg/ml diluted in 1X PBS
2. Gently tap the plate to make sure that the well 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 200µl 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 50µL PBS/0.05%tween-20(v/v) with 5% milk to all wells in rows B-H, columns 2-11.
7. Add 75µL of each diluted sample to the relevant wells in row A, columns 2-11.
8. Add 50µL 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 25µL 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 25µL 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 50µL 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 50µL TMB to all wells.
16. Allow to develop for 10 minutes.
17. Stop the reaction after 10 minutes by adding 50uL of 2M H_2SO_4 to all wells.
18. Read at 450nm 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 & SARS-CoV S glycoprotein [CR3022], Human IgM, Kappa (Absolute Antibody, Ab1680.15) dilute to 0.5µg/ml
- Anti-Human IgM (µ-chain specific) Peroxidase antibody produced in goat (Sigma #A0420) (use at 1 in 3000)

IgA

- Anti-COVID-19 & SARS-CoV S glycoprotein [CR3022], Human IgA, Kappa (Absolute Antibody, Ab1680.16) dilute to 0.5µg/ml
- Anti-Human IgA (α-chain specific) Peroxidase antibody produced in goat (Sigma #A0295) (use at 1 in 3000)

Appendix 6: Microneutralisation assay for Coronaviruses

Purpose

This SOP describes a method for quantifying the neutralising activity of antibodies against Coronaviruses (e.g. SARS-CoV-2, MERS-CoV, *etc*). 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.

Coronaviruses used for this assay must be listed in the FCS CL3 inventory. **Operators must have read and understood the BSDS associated with the pathogen in use.**

Associated documents

This SOP must be followed in conjunction with:
VIR/FCS/COP-BTP (workbench document 7349)
VIR/FCS/COP-for airborne pathogens (s/n 8582)
VIR/FCS/SOP 2: Operation of microbiological safety cabinets (workbench document 7379)
VIR/FCS/SOP3: Operation of centrifuge (workbench document 7379)
VIR/FCS/SOP 9: Operation of incubators (workbench document 7379)
VIR/FCS/SOP17: Decontamination and treatment of CL3 laboratory waste, equipment and clothing (workbench document 7379)
VIR/FCSCL3/Culturing of cell lines (workbench document 8154)
RA/03425: Use of the microbiology safety cabinets
RA/03172: Use of centrifuges
RA/03209: Transmitted Light and Fluorescent Microscopy
BSDS for the specific coronavirus in use (e.g. SARS-CoV-2)

Materials

Gilson p20, p200, p1000 pipette (or equivalent)
Multichannel pipette 20-200µl (or equivalent)
Pipette tips
Wypalls
Sealable secondary containers (eg sandwich box)
Tissue culture treated flat bottom (FB) 96-well plates (ThermoFisher: 10334791)
Sterile U bottom 96-well plate (ThermoFisher: 10520832)

Reagents

Choloros 10% in water
Microsol4 10% in water
Both to be prepared at the beginning of each day or session of work according to VIR/FCS/COP-BTP and VIR/FCS/SOP17)
70% IMS in water

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

- Growth medium – Dulbecco's MEM (Sigma, Cat No. D6546) or equivalent, supplemented with **10% foetal calf serum**, 2mM L-Glutamine (Sigma, Cat No. G7513) or equivalent e.g. Glutamax (Invitrogen, Cat. No. 35050-038) and 1% penicillin/streptomycin (Invitrogen cat no. 15140148)
- Dulbecco's MEM (Sigma, Cat No. D6546) or equivalent, supplemented with **4% foetal calf serum,** 2mM L-Glutamine (Sigma, Cat No. G7513) or equivalent e.g. Glutamax (Invitrogen, Cat. No. 35050-038) and 1% penicillin/streptomycin (Invitrogen cat no. 15140148)
- Trypsin/EDTA solution (Sigma, Cat No. T4049) or equivalent e.g. 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: 10010023); PBS-A from media can be used but it must be transferred in a plastic container;
- Washing Buffer - PBS/0.05%(v/v) Tween-20 (from media, transferred in a plastics container)- alternatively prepare by adding Tween-20 (Sigma cat no. P1379) to PBS
- 0.1% Triton-X100 (Sigma cat no. X100) diluted in 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 (from media)

<u>Antibodies- pathogen specific</u>

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

Procedure

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

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

Plates should be transported to/from MSC from/to incubator within sandwich boxes. Plates should be kept on a try 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 (e.g. -80°C and dry ice)

Day 1

This step can be done in a BSL2 or BSL3 laboratory.
If done in BLS3:

1. Turn on MSC and ensure appropriate checks are performed on MSC and recorded on log sheet prior to use.
2. Prepare the lab accordingly to lab CoP.

3. Seed VERO cells (CCL-81) at $2x10^4$ cells per well in a 96-well flat-bottom plate to achieve confluent monolayers the next day.

See VIR/FCS/Counting of cells (workbench document 8413)

4. Close the lids of the plates 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.

5. Place in a 37ºC, 5% CO_2 incubator overnight, opening box vent for gas exchange.

6. Alternatively, if this step is done in a BSL2 lab, the plates will need to be transported to room 3053 in the FCS, on the day of infection in a sealed container.

Day 2

Antibody dilutions can be done in BSL2 or BSL3.
If antibody dilutions have been undertaken in BSL2 (see point 14), prepared plates to be transported into room 3053 in the FCS in a sealed container.

All work with virus must be performed within the MSC

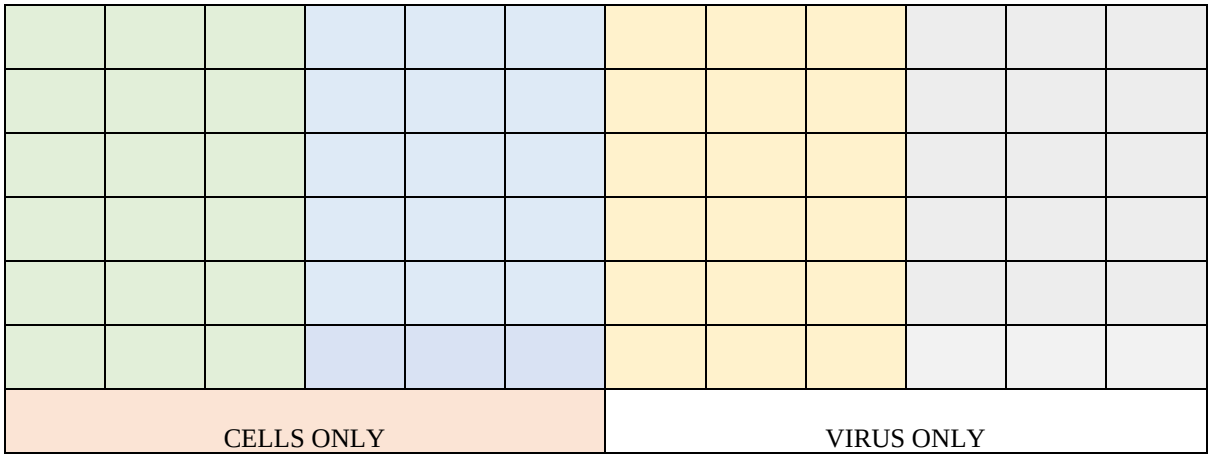
7. Before entering the BSL3 lab locate the viral stock on Item tracker.
8. Update storage records on ItemTracker.
9. Turn on BSL3 MSC and ensure appropriate checks are performed on MSC and recorded on log sheet prior to use.
10. Collect the virus stock within secondary container from storage and transport in secondary container to MSC.
11. Remove virus stock from container and place on a wypall soaked with 70% IMS to defrost.
12. Check the vial is defrosted and is not broken or leaking.
13. If the vial has leaked, then the sample should be disposed of as BSL3 waste.
14. Perform serial dilutions of the antibody samples in serum free media in a U- bottom 96-well plate, ideally in triplicate. Up to 4 antibody-containing samples can be assessed per plate (Figure 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

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

Sample 1	Sample 2	Sample 3	Sample 4



15. Dilute the virus stock in media without serum or antibiotics (e.g. MEM or DMEM) to add 60uL containing 100 TCID50 / well. (e.g. for a viral stock of $2x10^5$ TCID50/mL – $100 \div 2x10^5 = 0.5\mu L$ virus stock per well and 50uL virus stock diluted in 5,950µL serum free media to add 60µL containing 100 TCID50 to 100wells).
16. To each antibody dilution and positive control add 60 µL of diluted virus prepared as above.
17. 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.
18. Place in the incubator at 37ºC 5%CO_2 for 1hr.
19. Transfer plates with the virus/antibody dilutions and the plates seeded to the MSC within a sealed container.
20. Using a multichannel pipette, gently remove culture media from the plates with seeded cells.
21. Transfer 100µL of virus/antibody dilutions, positive and negative controls into each relevant well of the cell plate. An example of a potential layout is given in figure 1 above.

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

22. Label plates appropriately (virus name, antibody/sera name, date, dilution, user initials).
23. Using a multichannel pipette, add 100 µL of media with 4%FBS in each well.
24. 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 37ºC, 5% CO_2 incubator and open vent.
25. Incubate for 24 hours.

Day 3

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

26. Turn on MSC and ensure appropriate checks are performed on MSC and recorded on log sheet prior to use.
27. Prepare the lab according to CoP.
28. Retrieve plates within sealed container from the incubator and transport to the MSC.

29. Using a multichannel pipette, remove culture medium from the plates, and wash cells with 200µL PBS.
30. Add 200µL of 4% PFA (in PBS) to each well.
31. 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˚C).
32. Incubate the plate for more than 16 hours.

Day 4

33. Turn on MSC and ensure appropriate checks are performed on MSC and recorded on log sheet prior to use.
34. Prepare the lab according to CoP.
35. Retrieve plates from fridge and transport to MSC.

At this stage viral inactivation steps are complete and wash steps can continue by inversion of plate over wash box

36. Remove the PFA and wash once with 200µL PBS.
37. Add 150µL of 0.1% Triton-X100 (in PBS) to each well and incubate at room temperature for 15 minutes.
38. Remove with a multichannel pipette.
39. Wash plates once with 200µL of PBS-Tween (0.05%v/v).
40. Add 200µL of PBS-Tween (0.05%v/v)/3% Milk (blocking buffer) to each well and block for 1 hour at room temperature within MSC.
41. Wash plates 2 times with 200µL of PBS-Tween (0.05%v/v).
42. Add 50µl/well of the relevant primary antibody diluted in blocking buffer (e.g. anti-SARS-CoV-2 N protein diluted 1:2000).
43. Incubate plates for 1 hour at room temperature within the MSC.
44. During the incubation, remove TMB substrate from the fridge and warm to room temperature, protected from light.
45. Wash plates 3 times with 200µL of PBS-Tween (0.05%v/v).
46. Tap dry the plates on a Wypall, gently
47. Add 100µl of TMB substrate per well, and incubate for 5-15 minutes.
48. Stop the reaction with 100µL of 2N H2SO4.
49. 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
50. Read plates at O.D. 450nM

Appendix 7: Neutralisation Assay using SARS-CoV-2 Spike Lentiviral Pseudotyped Virus

SUMMARY

Pseudotyped virus-based neutralisation assays have been widely used as a surrogate for high-containment enveloped viruses, allowing greater accessibility to the study of virus entry inhibition by different biologicals. In many instances, it has been shown that neutralisation of the pseudotyped virus correlates with that of the live virus, including studies using SARS-CoV-1 and MERS-CoV pseudotyped virus (Temperton *et al.,* Emerging Infectious Diseases, 2005; 11(3); Perera *et al.*, Eurosurveillance, 2013; 18(36)). The system offers the advantage of being high-throughput and quantitative, with results acquired 48 hours after assay set-up by acquisition of reporter gene expression from target cells. This protocol describes a neutralisation assay using 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 neutralising activity of various biologicals such as serum, plasma and monoclonal antibodies.

MATERIALS

Cell Lines

- HEK-293T clone 17 cells (NIBSC CFAR catalogue: # 5016)

Culture Media

HEK-293T clone 17

- Gibco DMEM (1X) + GlutaMAX (ThermoFisher: # 61965-026)
- 10% v/v Fetal Calf Serum (Pan Biotech GmbH, P30-3306 Heat inactivated, South American origin)
- 1% v/v Penicillin-Streptomycin (Sigma-Aldrich: # P0781)

Plasmids/Recombinant Virus

- Expression plasmid: pCDNA3.1 hACE2 (Addgene: # 1786)
- Expression plasmid: pCSDest TMPRSS2 (Addgene: # 53887)

Reagents

- 0.25% Trypsin-EDTA Solution (Sigma-Aldrich: # T4049)
- Gibco Opti-MEM I (1X) (ThermoFisher: # 31985-047)
- Gibco DMEM (1X) Phenol Free (ThermoFisher: # 31053-028)
- FuGENE HD Transfection Reagent (Promega: # E2311)
- Bright-Glo Luciferase Assay System (Promega: # E2620)

Consumables/Equipment

- 10 cm TC-treated culture dish (Corning: # 430167)
- Falcon MicroWell TC-treated flat-bottom 96-well plate (ThermoFisher: #10334791)
- Falcon MicroWell TC-treated U-bottom 96-well plate (ThermoFisher: #10520832)

- Nunc F96 MicroWell White 96-well microplate (ThermoFisher: # 236108)
- Polypropylene sterile conical tubes, 15 mL (Sarstedt: # 62.554.502)
- 1.5 mL sterile micro-tubes (Sarstedt: # 72.692.005)
- GloMax Navigator Microplate Luminometer, or similar (Promega: # GM2000)
- Incubator at 37°C, 5% CO_2

PROCEDURE

All the work must be carried out within a microbiology safety cabinet (MSC) in a containment level 2 laboratory; operator at NIBSC must have read and understood the risk assessment for genetically modified microorganism work using non replicative, lentiviral vectors (GMM086 and GMM087).

Day 1: Seed Target Cells in Preparation for Transfection

1. Seed a 10 cm culture dish with $5x10^6$ HEK-293T/17 cells in 8mL culture media, to reach 60-80% confluence the next day. Typically, a single 10 cm dish yields enough cells for at least 7x 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 & Protease Expression Plasmids

1. Pre-warm to ambient temperature culture media 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:

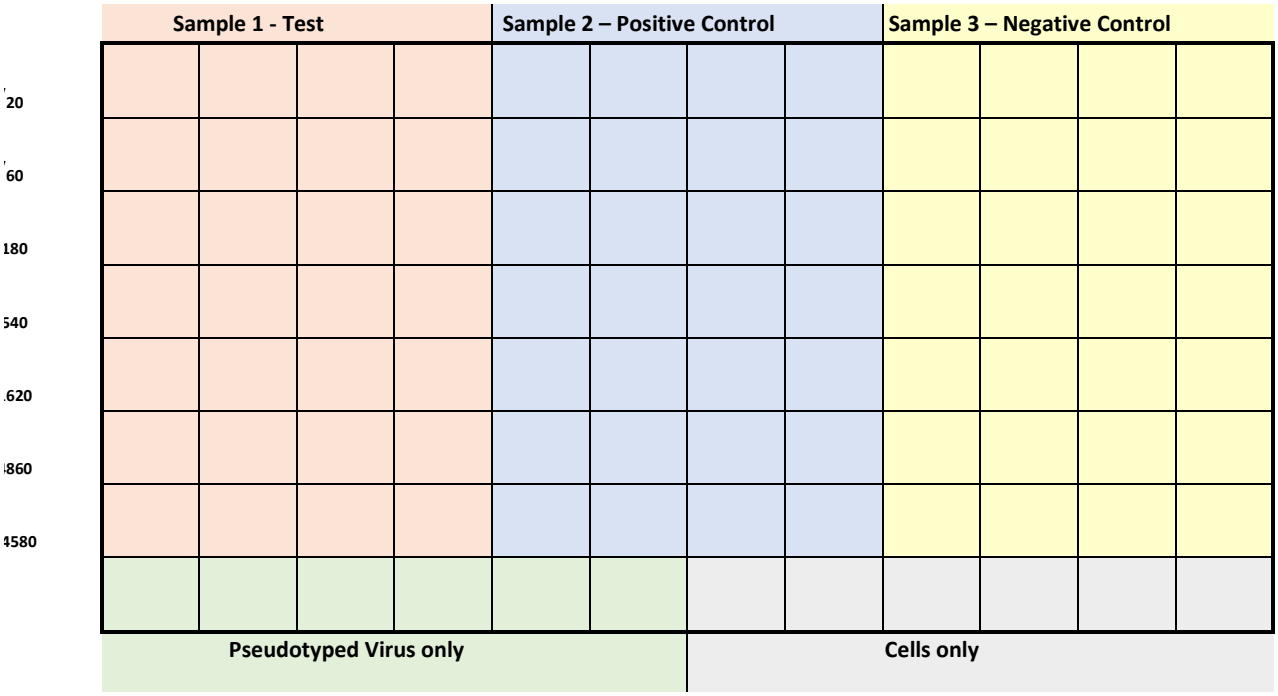
   2 µg pCDNA3.1 hACE2
   150 ng pCSDest TMPRSS2

3. Add 200 uL 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 within the MSC for 10-15 minutes
6. During the incubation, gently replace 8 mL culture media of the HEK-293T/17 cells seeded into a culture dish the previous day
7. Following incubation, add the transfection mix to the cell culture dish, dropwise while gently agitating plate to ensure even dispersal
8. Incubate at 37°C, 5% CO_2 for 24 hours

Day 3: Neutralisation of SARS-CoV-2 Spike Lentiviral Pseudotyped Virus

1. Following 24 hours incubation, remove media 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 media to $2x10^5$ cells/mL
3. Add 100 µL per well of a 96-well microplate, to give $2x10^4$ 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 with known neutralising activity and appropriate negative control sample each time an assay is performed
6. Calculate the amount of SARS-CoV-2 pseudotyped virus 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 pseudotyped virus and thaw at ambient temperature
8. In a 96-well sterile U-bottom plate, prepare a dilution series of each test sample, positive and negative control sample within a final volume of 60 µL culture media, taking into account a 1:2 dilution after the addition of pseudotyped virus at step 8. A 3-fold dilution series is recommended, performed at least in triplicate. Each plate should contain control wells of cells-only and pseudotyped virus-only.
   As per example layout below: add 81 µL culture medium into row A and 60 µL into all remaining wells. Next, add 9 µL serum into each well of row A, before performing a 3-fold serial dilution by carrying 30 µL across the rest of the dilution series rows B-G, discarding the final 30 µL.



9. Dilute SARS-CoV-2 pseudotyped virus in culture media to add 60 µL containing 150-300 $TCID_{50}$ to each well of the dilution plate except the cell-only controls, where 60 µL culture media is added to each well
10. Incubate at 37°C for 30 - 60 mins
11. Transfer 100 µL from each well of the dilution plate to the 96-well culture plate seeded with target cells in step 3, >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. 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 -77°C to -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 incubated for 5 mins ±2mins at room temperature (24°C ±4°C) to allow cell lysis
4. Mix gently 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, normalise the raw data to express results as percent neutralisation by defining 100% infectivity as the mean of cell-only wells and 0% infectivity as the mean of pseudotyped virus-only wells
7. Plot a graph of the average % neutralisation (*y*-axis) against the Log_{10} sample dilution (*x*-axis)
8. Fit a dose-dependent inhibition curve to the data via non-linear regression analysis to interpolate the IC_{50} values. It is recommended to perform this analysis in software such as GraphPad Prism®, with a detailed protocol available in Ferrara & Temperton, Methods and Protocols, 2018; 1(8), PMID: 31164554

Appendix 8: Preparation and calibration of national standard substances of biologics

I. Definition

The national standard substances of biologics refer to the biological standards or references used to determine potency, activity, or content of biological products, or used to identify and characterize biologics.

II. Classification of national standard substances

The 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. The content is expressed in milligram (mg). Biological activity is expressed in international units (IU), specific activity units (Arbitrary Units, AU) or in units (U).

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 qualitative identification of microorganisms (or its derivatives) or for disease reference materials used for the quantitative determination of biological potency of certain biological products, for example, reference materials used for titration of virus content in live measles vaccine, or of flocculation unites of toxoid, by which the potency can be expressed in specific activity units (AU) or in units (U) rather than in international units (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 the Chinese Good Manufacturing Practices for Pharmaceutical Products (GMP) or the Chinese Good Laboratory Practices (GLP).

2. The National Control Laboratory (NCL) is responsible for calibrating the national biological standard substances.

3. Research and development of new national standard substances

(1) Selection of source materials

The nature of source materials of national biological 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.

(2) Filling containers

Filling containers shall be neutral borosilicate glass. Heat sealing of the ampoule after the freeze-dried standard substances filled in will be good for stability of the standard substances.

(3) Formulation, filling, lyophilization and sealing of containers

Formulation and dilution of standard substances shall be performed as required. Necessary stabilizers or other materials shall not affect the activity stability and assaying processes of the standard substances, 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 the consistency of the potency and stability in each container during the course of filling, lyophilization and sealing.

(4) Test items

Test items shall be subject to the characters and purposes of the standard substances used, at least including the following tests but not limited to filling precision, residual moisture, sterility, biological activity/ potency and stability study.

(5) Calibration

① Collaborative calibration

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 the statistical analysis of the calibrated results shall be performed (the calibrated results necessitate at least five independent valid results).

② Confirmation of activity (potency unit or toxicity unit)

The activity is typically expressed as the mean value of the calibrated results obtained by participating laboratories. Data from collaborative calibration shall be collected and analyzed with statistical method by the NCL. Standard substances shall be given the activity value by using appropriate statistical analysis methods and official released after getting approval.

(6) Stability studies

The accelerated stability tests shall be performed during the development. Candidate substances shall be placed at various temperatures (-20℃, 4℃, 25℃ and 37℃) according to the characteristics for certain time for further testing of biological activity or content. The activity or content of established standard substances shall be checked periodically.

4. Preparation and calibration of a substitute lot of standard substances

(1) The NCL is responsible for the preparation and calibration

(2) Biological and 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 the standard substances

1. The collaboratively calibrated results of a newly established standard substance shall be reviewed and accepted by the NCL.

2. Substitute lot of standards substance shall be reviewed and accepted by the NCL.

3. The newly established standard substance shall be released for use only after obtaining approval.

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 condition, manufacturer, etc.

3. Package inserts shall be attached to standard substances and reference materials, and shall include the information in the labels, and in addition, the components and characters of the substance/material, usage method, stability, etc. If necessary, the references shall be provided.

VI. Use, release and storage of standard substances

1. National standard substances of biologics shall apply for implementing national standards for drugs. The valuation of the national standard substances of biologics shall be valid only within the specified usage. If applied for other purpose, its applicability shall be

confirmed by user themselves.

2. Requests for national standards substances of biologics should be made directly to the NCL. The national standard substances are provided to manufacturers to calibrate working standards or for quality control purpose.

3. National standard substances shall be stored at an appropriate temperature and humidity, which shall be periodically monitored and recorded.

A designated person shall be responsible for managing and releasing national standard substances of biologics.

Appendix 9 Calibrating SARS-CoV-2 Immunoassay Internal Assay Reference Reagents to International Standards and/or Secondary Standards

1. PURPOSE

This Guidance Document is designed to describe the calibration procedure when using International standards, secondary standards, and/or internal assay reference reagents.

2. SCOPE

This Guidance Document applies to all SARS-CoV-2 immunoassays requiring calibration of internal assay reference reagents to a secondary standard and/or international standard.

3. REFERENCES

3.1. Recommendations for the preparation, characterization and establishment of international and other biological reference standards (revised 2004), World Health Organization, WHO Technical Report Series, No. 932, 2006.

3.2. Application Note, Parallel line analysis and relative potency in SoftMax Pro 7 Software, 2016 Molecular Devices, LLC.

3.3. Gottschalk, P.D. and Dunn, J.R. 2005. Measuring parallelism, linearity, and relative potency in bioassay and immunoassay data. Journal of Biopharmaceutical Statistics 15(3): 437-463.

3.4. Bates D. M. and Watts D. G. 1988. NonLinear Regression Analysis and its Applications. New York, Wiley.

4. DEFINITIONS

Calibrator: Material, biological, such as antibodies, in nature that 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.

5. PROCEDURE PRINCIPLES

Assign an International unit per milliliter (IU/mL) or binding antibody unit per milliliter (BAU/mL) to an internal assay reference reagent that is used daily in an assay. The unit is dependent on the type of calibrator being used, an international standard (primary standard) would have International units (IU) assigned, while a secondary standard would have another

unit assigned such as arbitrary units, mg, index value, unless it has been calibrated to the international standard, then the units of the secondary standard would be IU/mL.

6. PROCEDURE

Test the calibrator (WHO IS or Secondary Standard [if the WHO IS is not available]) in triplicate (independent serial dilutions) in the same plate as the internal assay reference reagent (ex. daily assay standard). Perform serial dilutions of the calibrator, so the calibrator reaches end point dilution/titer/concentration. For consistency, the fold dilution of the calibrator should match the fold dilution of the internal assay reference reagent (ex. 2- fold or 3-fold serial dilutions).

Figure 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

In Figure 1, a representative plate map design for an immunoassay is depicted, yet alternative schemes may be used to suit the assay. Figure 1 highlights the use of serial dilutions of each sample and to test each sample with at least three replicates. Assay controls per standard operating procedure should be included in each plate to verify system suitability. Perform the test on three separate days in the exact manner and set up as performed 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 for 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.

7. 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 COTS (commercial off the shelf) programs that can perform parallel line analysis, and the analysis can also be completed in R. Parallelism methods may 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.

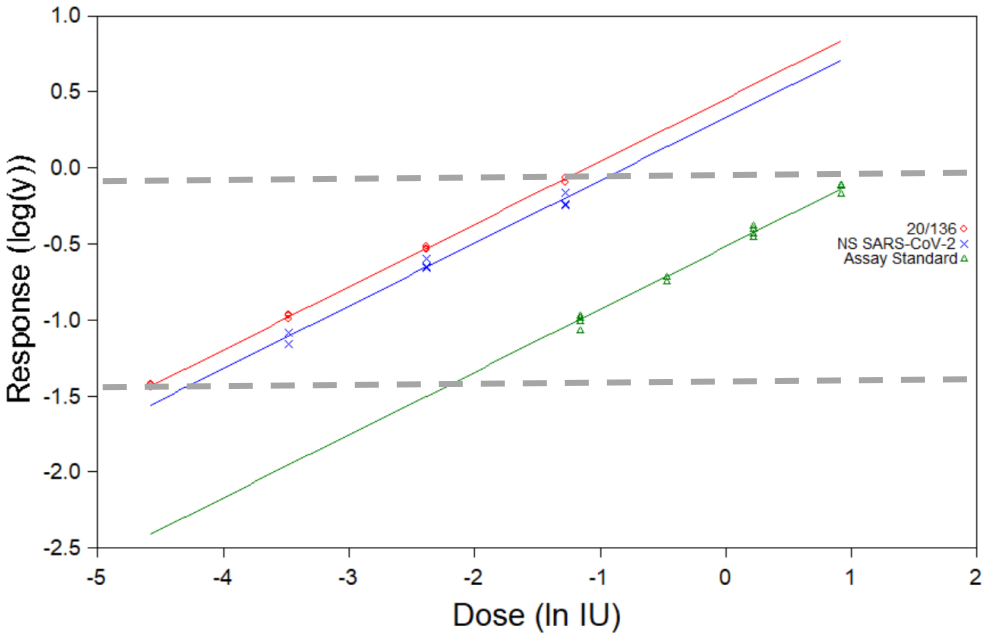
The calibrator is treated as the reference, and the potency value for the calibrator may be found on the respective Instructions for Use document. For reference, Table 1 describes the unitage of the WHO anti-Human SARS-CoV-2 Serology Standard (20/136) when reconstituted following instruction for use.

Table 1. Assigned Neutralizing and Binding Unitage of WHO anti-Human SARS-CoV-2 International Standard (20/136).

	WHO anti-Human SARS-CoV-2 Serology Standard (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

In Figure 2, the Dose (ln IU) vs. Response (log(y)) is graphed for the WHO Human SARS-CoV-2 Serology Standard (20/136, red circles), National Human SARS-CoV-2 Serology Standard (blue x), and internal assay reference reagent (green triangle). Of note when reviewing the plots, verify 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 gray dotted lines in Figure 2) so as not to perform analysis on extrapolated data. Combistats allows the analyst to assign a relative potency value to the calibrator, and the program performs the relevant calculations needed to determine if the samples (National Human SARS-CoV-2 Serology Standard and internal assay reference reagent) are parallel to the calibrator (WHO Human SARS-CoV-2 Serology Standard (20/136)) as depicted in Figure 2. Next, the analyst will review the probability of the Dose (ln IU) vs. 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 (ln IU) vs. Response (log(y)) lines are parallel and linear. Furthermore, the Combistats program calculates the relative potency of the samples, and this value in turn will be used to calculate the potency of the sample across the three days of testing.

Figure 2. Parallelism graph for Spike IgG assay using Combistats



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 value from each of the three days is calculated, and the geometric mean value represents the final potency (calibrated) value for each sample. Table 2 illustrates the calibration process with Combistats using representative data.

Table 2. Representative data to illustrate the calibration process with a quantitative assay using Combistats.

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

C_STD: Internal Assay Reference Reagent
STD-C: Calibrator
STD-T: Secondary Standard

Calibration calculations:
Assumptions: Calibrator equals 1000 BAU/mL
C_STD: 93 BAU/mL
STD-T: 735 BAU/mL

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

Table 3. Representative data to illustrate the calibration process with 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				

C_STD: Internal Assay Reference Reagent
STD-C1, C2, and C3: Calibrator
STD-T1, T2, and T3: Secondary Standard

Calibration calculations:

Assumptions: Calibrator equals 1000 IU/mL
C_STD- (1000 IU/mL / 969 Titer) * 2983 Titer = 3078 IU/mL
STD-T- (1000 IU/mL / 969 Titer) * 522 Titer = 539 IU/mL

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

Appendix 10: Calibrating HPV Immunoassay Internal Assay Reference Reagents to International Standards and/or Secondary Standards (Guidance Document)

1. PURPOSE

This Guidance Document is designed to describe the calibration procedure when using HPV International standards, secondary standards, and/or internal assay reference reagents.

2. SCOPE

This Guidance Document applies to all HPV immunoassays requiring calibration of internal assay reference reagents to a secondary standard and/or international standard.

3. REFERENCES

a. Recommendations for the preparation, characterization and establishment of international and other biological reference standards (revised 2004), World Health Organization, WHO Technical Report Series, No. 932, 2006.

b. Application Note, Parallel line analysis and relative potency in SoftMax Pro 7 Software, 2016 Molecular Devices, LLC.

c. Gottschalk, P.D. and Dunn, J.R. 2005. Measuring parallelism, linearity, and relative potency in bioassay and immunoassay data. Journal of Biopharmaceutical Statistics 15(3): 437-463.

d. Bates D. M. and Watts D. G. 1988. NonLinear Regression Analysis and its Applications. New York, Wiley.

4. DEFINITIONS

Calibrator: Material, biological, such as antibodies, in nature that 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.

5. PROCEDURE PRINCIPLES

Assign an International unit per milliliter (IU/mL) or binding antibody unit per milliliter (BAU/mL) to an internal assay reference reagent that is used daily in an assay. The unit is dependent on the type of calibrator being used, an international standard (primary standard) would have International units (IU) assigned, while a secondary standard would have another

unit assigned such as arbitrary units, mg, index value, unless it has been calibrated to the international standard, then the units of the secondary standard would be IU/mL.

6. PROCEDURE

Test the calibrator (WHO IS or Secondary Standard [if the WHO IS is not available]) in triplicate (independent serial dilutions) in the same plate as the internal assay reference reagent (ex. daily assay standard). Perform serial dilutions of the calibrator, so the calibrator reaches end point dilution/titer/concentration. For consistency, the fold dilution of the calibrator should match the fold dilution of the internal assay reference reagent (ex. 2- fold or 3-fold serial dilutions).

Figure 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

In Figure 1, a representative plate map design for an immunoassay is depicted, yet alternative schemes may be used to suit the assay. Figure 1 highlights the use of serial dilutions of each sample and to test each sample with at least three replicates. Assay controls per standard operating procedure should be included in each plate to verify system suitability. Perform the test on three separate days in the exact manner and set up as performed 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 for 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.

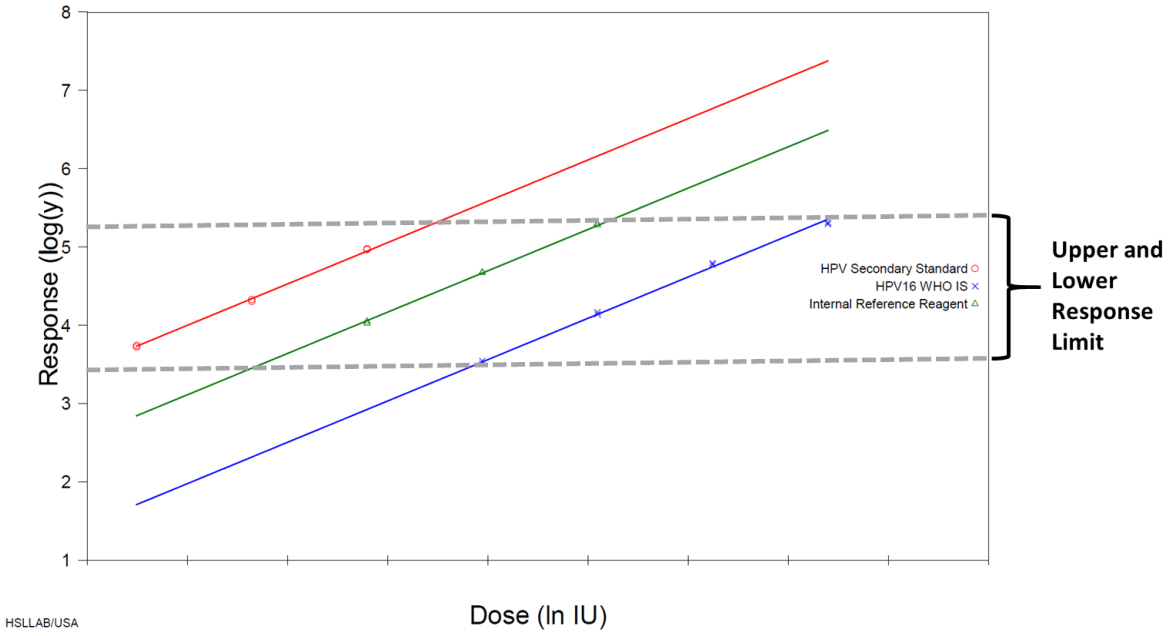
7. DATA ANALYSIS

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 COTS (commercial off the shelf) programs that can perform parallel line analysis, and the analysis can also be completed in R. Parallelism methods may 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 on the respective Instructions for Use document.
In Figure 2, the Dose (ln IU) vs. Response (log(y)) is graphed for the HPV Secondary Standard (red circles), WHO HPV-16 International Standard (blue x), and internal assay reference reagent (green triangle). Of note when reviewing the plots, verify 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 gray dotted lines in Figure 2) so as not to perform analysis on extrapolated data. Combistats allows the analyst to assign a relative potency value to the calibrator, and the program performs the relevant calculations needed to determine if the samples (HPV Secondary Standard and internal assay reference reagent) are parallel to the calibrator (WHO HPV-16 International Standard)) as depicted in Figure 2. Next, the analyst will review the probability of the Dose (ln IU) vs. 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 (ln IU) vs. Response (log(y)) lines are parallel and linear. Furthermore, the Combistats program calculates the relative potency of the samples, and this value in turn will be used to calculate the potency of the sample across the three days of testing.

Figure 2. Parallelism graph for Anti-HPV-16 IgG assay using Combistats

Note: Data in Figure 2 is hypothetical and is used 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 value from each of the three days is calculated, and the geometric mean value represents the final potency (calibrated) value for each sample. Table 1 illustrates the calibration process with Combistats using representative data.

Table 1. Representative data to illustrate the calibration process with a quantitative assay using Combistats.

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

C_STD: Internal Assay Reference Reagent

STD-C: Calibrator
STD-T: Secondary Standard

Calibration calculations:

Assumptions: Calibrator equals 10 IU/mL
C_STD: 93 IU/mL
STD-T: 735 IU/mL
Note: Data in Table 1 is hypothetical and is used for illustration purposes.

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

Table 2. Representative data to illustrate the calibration process with 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				

C_STD: Internal Assay Reference Reagent
STD-C1, C2, and C3: Calibrator
STD-T1, T2, and T3: Secondary Standard

Calibration calculations:

Assumptions: Calibrator equals 1000 IU/mL
C_STD- (1000 IU/mL / 969 Titer) * 2983 Titer = 3078 IU/mL
STD-T- (1000 IU/mL / 969 Titer) * 522 Titer = 539 IU/mL
Note: Data in Table 2 is hypothetical and is used for illustration purposes.

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