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WHO Expert Committee on Biological Standardization

Seventy-fifth report

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WHO Expert Committee on Biological Standardization: seventy-fifth report

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Seventy-fifth meeting held virtually 4 to 8 April 2022

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Abbreviations

ACT	WHO Access to COVID-19 Tools (Accelerator)
Ag-RDT	antigen-detecting rapid diagnostic test
ASP	antiphospholipid syndrome
β2GPI	β2 glycoprotein I
BAU	binding antibody unit(s)
COVID-19	coronavirus disease 2019
ddPCR	droplet digital polymerase chain reaction
DNA	deoxyribonucleic acid
EEA	European Economic Area
EFI	European Federation for Immunogenetics
ELISA	enzyme-linked immunosorbent assay
EV-D68	enterovirus D68
EU	European Union
EUA	(US Food and Drug Administration) emergency use authorization
EUL	(WHO) emergency use listing
HLA	human leukocyte antigen
HNA	human neutrophil antigen
HPV	human papillomavirus
HTS	high-throughput sequencing
IFFC	International Federation of Clinical Chemistry and Laboratory Medicine
IFU	instructions for use
IgG	immunoglobulin G
ISBT	International Society of Blood Transfusion
ISTH	International Society on Thrombosis and Haemostasis
IU	International Unit(s)
IVD	in vitro diagnostic

JRC	Joint Research Centre (of the European Commission)
LASV	Lassa virus
LV	lentiviral vector
mAb	monoclonal antibody
MAT	monocyte activation test
NAT	nucleic acid amplification technique
NC3Rs	National Centre for the Replacement, Refinement & Reduction of Animals in Research
NCL	national control laboratory
NEP	non-endotoxin pyrogen
NIBSC	National Institute for Biological Standards and Control
NRA	national regulatory authority
PCR	polymerase chain reaction
PEI	Paul-Ehrlich-Institut
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
RPT	rabbit pyrogen test
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
TLR	toll-like receptor
TRALI	transfusion-related acute lung injury
VOC	variant of concern

1. Introduction

The seventy-fifth meeting of the WHO Expert Committee on Biological Standardization was held virtually from 4 to 8 April 2022. The meeting was opened on behalf of the Director-General of WHO and the Assistant Director-General, Access to Medicines and Health Products, by Dr Clive Ondari, Director, Health Products Policy and Standards. Dr Ondari began by welcoming Committee members, meeting participants and observers, and noted that the Expert Advisory Panel on Biological Standardization, from which the Committee was drawn, had now been expanded to more than 20 members. This expansion was part of an ongoing effort by WHO to ensure that the appropriate breadth of expertise was available for the increasingly diverse range of biological medicines now being considered by the Committee, and to ensure the geographical representativeness of its advisory groups.

Since 2020, the work of the Committee had been dominated by standardization issues arising from the coronavirus disease 2019 (COVID-19) pandemic, and Dr Ondari highlighted the crucial importance of harmonized bioassays for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in supporting WHO prequalification and emergency use listing (EUL) of products for COVID-19 diagnosis, prevention and treatment. He also highlighted the importance of WHO written standards in this area, including the recently published WHO guidance on regulatory considerations in evaluating the quality, safety and efficacy of messenger RNA vaccines for the prevention of infectious diseases. The Committee was informed that WHO had recently updated its strategic plan for COVID-19 to reflect the development of new vaccines against SARS-CoV-2 variants of concern (VOCs), which would have implications for the development of future WHO measurement standards. Dr Ondari noted, however, that despite the focus placed on COVID-19 diagnostics, vaccines and therapeutics over the past 2 years, work had also continued on a number of important non-COVID-19 projects, and Dr Ondari expressed his thanks to WHO staff for dealing with the additional workload so effectively.

Turning to the agenda for the current meeting, Dr Ondari highlighted the continuing contribution of the work of the Committee to the three pillars of the WHO Access to COVID 19 Tools (ACT) accelerator – noting in particular the prospective WHO Guidelines for the production and quality control of monoclonal antibodies and related products intended for medicinal use, and WHO manual on the preparation of secondary standards for use in antibody testing. Furthermore, the proposed revised WHO Guidelines on evaluation of biosimilars would directly contribute to the WHO goal of increasing access to these key products, in full accordance with World Health Assembly resolution WHA67.21. In addition to consideration of these WHO written standards,

proposals and updates would be also presented on a range of WHO measurement standards, including updates on the rapid development of WHO measurement standards for SARS-CoV-2. Such standards will be key in the context of ongoing WHO ACT accelerator efforts, and WHO greatly appreciated the invaluable and ongoing efforts of the United Kingdom's National Institute for Biological Standards and Control (NIBSC) in this regard.

Reflecting on the preference expressed by Committee members for face-to-face meetings, Dr Ondari thanked everyone for their forbearance with regard to the recent virtual meetings necessitated by the COVID-19 pandemic. Should the epidemiological situation permit, consideration would be given to adopting a hybrid approach for the next meeting of the Committee in October 2022 in which some participants would participate virtually while others would meet in Geneva.

Dr Ivana Knezevic, Secretary to the Committee, thanked Dr Ondari for his opening remarks. She reminded participants that, as a specialized agency of the United Nations, WHO is mandated to direct and coordinate international public health matters on behalf of its 194 Member States. In this regard, WHO is expected to provide leadership on global health matters, shape the health research agenda, set norms and standards, articulate evidence-based policy options, provide technical support to countries, and monitor and assess health trends. Setting norms and standards and promoting their implementation is therefore a core WHO function and in the context of the ongoing COVID-19 pandemic – which had brought into sharp focus the challenge of attaining the highest level of health for all – a wide range of biological standardization initiatives had been presented to the Committee for its consideration. Dr Knezevic noted that the upcoming meeting of the World Health Assembly, the decision-making body of WHO attended by delegates from all Member States, highlighted the importance of regularly reporting upon the activities of the Committee to the Health Assembly's Executive Board.

Dr Knezevic then explained that, at least in the short term, meetings of the Committee would be held twice a year – with a virtual meeting in April to allow for the timely endorsement or establishment of WHO measurement standards, and consideration of upcoming WHO written standards, followed by the principal face-to-face meeting in October. Dr Knezevic expressed her thanks to WHO staff, WHO drafting and working group members, colleagues from WHO collaborating centres and custodian laboratories, and the many individual experts involved in the development and/or revision of WHO written standards and establishment of WHO measurement standards.

Dr Knezevic went on to outline the meeting procedures and working arrangements. An open information-sharing session involving all participants including non-state actors would be held on Monday 4 April 2022. Committee members, regulatory authority representatives and subject matter experts from

governmental organizations would then participate in the main meeting from Monday 4 April to Thursday 7 April 2022. All final decisions and recommendations on the adoption of WHO written standards and the establishment of WHO measurement standards would be made in a closed session held on Friday 8 April attended only by Committee members and the WHO Secretariat.

Following the conclusion of the open information-sharing session, the meeting officials were elected. In the absence of dissent, Dr Celia Witten was elected as Chair with Professor Klaus Cichutek and Dr Salwa Hindawi as Vice-chairs. Dr Ian Feavers and Dr Diana Teo were elected as Rapporteur and Co-rapporteur respectively. Dr Knezevic presented the declarations of interests completed by all members of the Committee, and by WHO temporary advisers and other participants. After evaluation, WHO had concluded that none of the interests declared constituted a significant conflict of interest and that the individuals concerned would be allowed to participate fully in the meeting.

The Committee then adopted the proposed agenda and timetable (WHO/BS/2022.2422).

2. General

2.1 Strategic directions in biological standardization

2.1.1 COVID-19 standardization: lessons learnt and vision for the future

Dr Mark Page reviewed the challenges of developing urgently needed measurement standards during the COVID-19 pandemic and proposed a number of steps to facilitate the rapid development of standards during future public health emergencies. Unprecedented global demand for the First WHO International Standard for anti-SARS-CoV-2 immunoglobulin, established in December 2020, had resulted in its depletion by August 2021, with more than 2400 units shipped to 581 individual users. However, this rate of attrition was caused primarily by the misuse of the reference material for assay validation rather than calibration, with less than half of users reporting their results in International Units (IU). A key factor in this had been the lack of secondary standards early in the pandemic. This had been largely inevitable as such standards take time to produce and suitable source material is typically unavailable at the start of an outbreak.

Barriers to the adoption of an IU generally include: (a) a lack of clarity among some users regarding assay calibration, which in the case of the First WHO International Standard for anti-SARS-CoV-2 immunoglobulin for use in neutralization assays had been compounded by its simultaneous recommended use as an interim NIBSC research reagent with assigned binding antibody units (BAU) for antibody binding assays – resulting in issues for serology standards used for both vaccine response evaluations and diagnostics; (b) inconsistent use of internal assay standards; and (c) the unavailability of WHO international standards when assays are first developed, particularly during a pandemic when assays are developed at pace. Addressing these and other end user challenges would require improving understanding of the purpose of WHO international standards and related reference materials. Recent WHO activities in this area include the development of the WHO manual for the preparation of reference materials for use as secondary standards in antibody testing, scheduled for discussion at the current meeting (see section 3.1.1 below). It is intended that the guidance provided will support end users of WHO international standards by reinforcing best practices in the calibration and use of antibody secondary standards. It is anticipated that training webinars and workshops will be held in conjunction with publication of the manual.

Dr Page went on to note that one important lesson learnt from the COVID-19 pandemic had been that due to the time required for antibody titres to develop in convalescent plasma, the immediate production and evaluation of an international standard was not feasible. As a result, research reagents had provisionally been used as reference materials while the WHO international standard was being prepared. One such antibody research reagent had been

made available by NIBSC in April 2020 and subsequently calibrated as part of the collaborative study to establish the WHO international standard to allow laboratories to retrospectively calibrate their assays to the WHO international standard. The pandemic had also highlighted the lack of a coordinated framework for sourcing bulk materials for preparing standards – a problem compounded by the emergence of VOCs and the changing vaccination status of potential donors. In response, WHO had now initiated development of the WHO BioHub for sharing non-influenza biological materials with epidemic or pandemic potential. Dr Page then summarized the outcomes of the recent HARMONY study into how well commercially available antibody binding assays had been harmonized by external reference reagents, and how well internal laboratory standards had been calibrated. In addition to the various commercial references and calibrators provided with the assays, the study had also included the First WHO International Standard for anti-SARS-CoV-2 immunoglobulin, along with secondary reference materials from the Frederick National Laboratory for Cancer Research in the USA and NIBSC in the United Kingdom. Although valid estimates were obtained from all assays, with low inter-run variability, the degree of assay harmonization was found to be antigen-target dependent, with significant differences also noted between the American and British reference materials. Additional assays would now be evaluated and the analysis finalized prior to reporting to manufacturers. Dr Page concluded by presenting a number of potential next steps in relation to the WHO BioHub, preparedness for emerging pathogens, the development and use of reference materials and the need for improved responsiveness during public health emergencies.

During discussion, the Committee acknowledged the importance of repository initiatives such as the WHO BioHub, as well as the role of funding organizations, in providing a preparedness framework for both known priority pathogens and novel infections. It was also recognized that holding biannual meetings of the Committee to establish new standards and reference panels would improve responsiveness in this area. Meeting participants commented that although many countries were now producing serological reagents for SARS-CoV-2 not all were aware of the WHO international standard, and it was suggested that WHO regional offices might help to promote its use. Noting that WHO was already working with its regional offices and networks of national regulators to raise awareness of the international standard, the Committee proposed that during public health emergencies information-sharing should be streamlined and less hierarchical to ensure that all stakeholders receive critical information in a timely manner. The Committee also discussed possible explanations for the differences observed in the HARMONY study data for the American and British secondary reference materials. However, noting that the plasma pools used to produce both materials had been obtained from unvaccinated individuals convalescing from

infection with the original Wuhan strain of SARS-CoV-2, it was concluded that there was insufficient information at present to provide a clear explanation.

2.1.2 Priorities in the development or revision of WHO written standards

Dr Knezevic reviewed current WHO priorities for the development of new or revised WHO written standards for biological products. Although not all recent and upcoming WHO written standards were of relevance to COVID-19, the pandemic had unavoidably led to the de-prioritization of several documents during the last 2 years. Recently published WHO written standards applicable to COVID-19 vaccines, therapeutics and diagnostics included the WHO Guidelines on the quality, safety and efficacy of plasmid DNA vaccines, and a WHO guidance document on regulatory considerations in evaluating the quality, safety and efficacy of messenger RNA vaccines, adopted in 2020 and 2021 respectively. In addition, the WHO manual for the preparation of reference materials for use as secondary standards in antibody testing, and the WHO Guidelines for the production and quality control of monoclonal antibodies and related products intended for medicinal use, would be considered for adoption at the current meeting (see sections 3.1.1 and 3.2.2 below, respectively). It was further intended that WHO Guidelines on the nonclinical and clinical evaluation of monoclonal antibodies used for the prophylaxis and treatment of infectious diseases would be presented for consideration by the Committee in March 2023. Consideration was currently being given to the development of supplemental disease-specific guidance to this document – for example on SARS-CoV-2 and respiratory syncytial virus – with similar supplemental guidance on rabies, malaria and HIV to potentially be drafted subsequently.

Dr Knezevic then outlined a number of non-COVID-19-related WHO written standards that the Committee had previously recommended for updating in light of recent scientific and technological advances. Among these, the WHO Recommendations to assure the quality, safety and efficacy of poliomyelitis vaccines (oral, live, attenuated) was currently being revised with a view to presenting it to the Committee for its consideration in October 2022. The revised document will incorporate the development of novel oral poliomyelitis vaccine production strains and innovative quality control technologies such as high-throughput sequencing (HTS) – while also taking into account the requirements of WHO EUL and GAPIII.[7] In addition, despite their amendment in October 2021, the WHO Recommendations to assure the quality, safety and efficacy of live attenuated yellow fever vaccines now require complete revision to reflect

[7] WHO global action plan to minimize poliovirus facility-associated risk after type-specific eradication of wild polioviruses and sequential cessation of oral polio vaccine use. GAPIII. Geneva: World Health Organization; 2015 (https://apps.who.int/iris/handle/10665/208872, accessed 13 June 2022).

the development of cell-based production methods, the application of HTS for quality control and the ongoing development of WHO international standards. Other WHO written standards requiring fundamental revision include the WHO Guidelines to assure the quality, safety and efficacy of live attenuated rotavirus vaccines (oral), and the now outdated WHO Requirements for measles, mumps and rubella vaccines and combined vaccine (live). During 2023, the current WHO guidelines on malaria vaccines and on dengue vaccines will also be reviewed to identify required revisions. Depending on the outcomes of ongoing vaccine developments in the respective fields, new WHO guidelines may also be required for non-typhoid Salmonella, Shigella and group B streptococcus, while new or revised WHO guidelines may similarly be required for tuberculosis vaccines.

In addition to the above disease-specific documents, a number of general WHO documents also now required revision. Among these, the WHO Recommendations for the preparation, characterization and establishment of international and other biological reference standards had been identified by the Committee as in need of updating. This document will now be reviewed during 2022–2023 with a view to developing two separate documents – one to provide guidance to custodian laboratories and the other for end users of such standards. As part of regulatory systems strengthening efforts, the WHO Global Model Regulatory Framework for Medical Devices including in vitro diagnostic medical devices is also undergoing revision to expand upon a number of important topics and address new issues that had arisen since its adoption and publication in 2017. It was envisaged that the revised document would be submitted to the Committee for its consideration in October 2022. Dr Knezevic concluded by highlighting an upcoming WHO guidance document on the regulatory convergence of cell and gene therapy products that was also scheduled for submission to the Committee in October 2022.

Recognizing the importance of WHO COVID-19 written standards in the regulatory evaluation of pandemic vaccines worldwide and the challenges presented by VOCs, the Committee wondered whether WHO guidance should now be revised to accommodate and potentially simplify the evaluation of second-generation vaccines that incorporated additional or alternative strains. The Committee was assured that this would be addressed as part of updated WHO strategic plans for responding to the COVID-19 pandemic.

2.1.3 Update on the 3Rs project

Dr Richard Isbrucker updated the Committee on an ongoing review of the animal testing requirements and procedures set out in publicly available WHO written standards for vaccines and biotherapeutics. The purpose of the review was to determine which tests are currently recommended for the quality control and lot release of such products, and whether strategies based on the 3Rs principles

of animal testing ("Replace, Reduce, Refine") were now available that are not included in existing WHO guidance. The review is also exploring the barriers to the adoption of 3Rs principles by NRAs, national control laboratories (NCLs) and manufacturers, and will ultimately inform WHO strategies for facilitating the consistent implementation of the principles. The scope of the review process does not include the development or validation of any 3Rs methods. As the review is intended to provide recommendations based on a sound scientific rationale, it also does not include consideration of the ethical aspects of animal use in quality control or lot release testing.

The 3-year project is expected to report to the Committee in October 2023 and is divided into two stages. The first stage, to review WHO written standards and make recommendations, was being led by the National Centre for the Replacement, Refinement & Reduction of Animals in Research (NC3Rs) in the United Kingdom, which will also deliver the final report. This approach is intended to avoid any perception of bias that might arise were WHO to review its own documents. The second stage, the course of which will be dependent upon the outcomes of the first stage, will be a response and implementation phase led by WHO and guided by the advice of the Committee.

Dr Elliot Lilley continued the update by providing a brief overview of NC3Rs and of its approach to the first stage of the project. Funding for this stage had been secured from the Bill & Melinda Gates Foundation and NC3Rs, and an international working group had been established that included experts representing manufacturers and regulators. To date, 81 WHO documents had been reviewed, 63 of which referred to animal methods related to lot release testing. Following this review, the working group suggested that the language used in future WHO guidance should emphasize the scientific benefits of the 3Rs principles. A lack of consistency was also noted between older and newer documents – for example, with regard to the variable advice provided on pyrogen and endotoxin testing, and particularly the language pertaining to the 3Rs principles and animal testing. Based on the findings of the review, five focus groups had been established to evaluate the potential for adoption of the 3Rs principles in testing for: (a) adventitious agents; (b) neurovirulence; (c) potency/immunogenicity; (d) pyrogenicity/endotoxin content; and (e) specific toxicity. It was intended that each group would propose revised and consistent texts in these areas that emphasize the scientific benefits of the 3Rs principles, non-animal alternatives, optimized experimental design and high standards of animal welfare, based on robust scientific evidence. Animal tests will only be recommended for deletion from WHO written guidance where there is a sound scientific basis for doing so. Regular stakeholder engagement is being maintained throughout the project and regional workshops were being held online to inform project recommendations and their implementation by WHO.

Dr Lilley then presented the results of a survey conducted among manufacturers that had elicited 28 responses from 14 countries. Analysis of the responses had shown that although most manufacturers knew that WHO had removed the requirement for the general safety (abnormal toxicity) test, more than half still performed it. Furthermore, although the majority of manufacturers recognized the importance and potential benefits of the 3Rs principles, concerns were cited over a perceived failure to meet local regulatory requirements as a critical barrier to adopting them. Going forwards, manufacturers rated the revision of WHO guidance on the 3Rs principles, along with supportive WHO statements, as key factors in supporting their implementation. A survey of NRAs and NCLs had been conducted in early 2022 and responses were now being collated. Four regional workshops and additional stakeholder meetings were also planned for 2022. Dr Lilley concluded by asking the Committee if there might be benefit in WHO giving consideration to the drafting of a position statement on the incorporation of 3Rs principles into lot release testing, along with a manual specifically on endotoxin and pyrogen testing.

The Committee felt that this review of animal testing requirements in WHO written documents was an interesting and valuable undertaking. It agreed that there was a need to emphasize the scientific benefits of the 3Rs principles, while also recognizing the challenges in validating alternative in vitro methods. The Committee felt that developing a WHO position statement would be premature until the Committee had had an opportunity to review the final report of the first phase of the project. It was further agreed that a comprehensive table of the animal tests currently in use would be a very useful addition to the report. The Committee was assured that such a table would be produced and would indicate alternative methods, new technologies with potential, and cases where a call for the development of new methods would be appropriate. While acknowledging that the information document provided at the current meeting was a work in progress, the Committee advised that the final texts produced by the five focus groups should be clear and consistent to prevent their variable interpretation by users.

3. International Recommendations, Guidelines and other matters related to the manufacture, quality control and evaluation of biological products

3.1 General

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

The development, establishment and promotion of international reference standards for ensuring the quality and consistent dosing of biological medicinal products used worldwide is a core function of WHO. WHO international standards are assigned a value in IU and serve as the primary measurement standards for the calibration of national and other secondary standards. Such secondary standards are frequently developed by NCLs and manufacturers to meet specific needs and to reduce demand for the corresponding WHO international standard, which may be in limited supply.

In addition to the WHO Recommendations for the preparation, characterization and establishment of international and other biological reference standards (revised 2004), WHO has also published two manuals on the development of secondary standards specifically used for the evaluation of vaccines and in vitro diagnostics. Following feedback received by WHO in 2020, a need was identified for a corresponding WHO manual on the preparation, calibration and use of secondary standards used in antibody testing. The need for such a manual was then reinforced by the unprecedented level of demand for the First WHO International Standard for anti-SARS-CoV-2 immunoglobulin, as well as by requests for antibody standards to support the evaluation of biological products for the prevention and treatment of other infectious diseases.

The Committee was provided with an overview of the structure and content of the proposed document and of the document development process, which had included two rounds of public consultation. The drafting group had agreed that although the manual should focus on the development of secondary standards for the evaluation of antibody responses to SARS-CoV-2, it should also be sufficiently flexible to provide broadly applicable guidance on the development of antibody secondary standards for any infectious disease. In addition, a section had been included on the appropriate use of biological standards to highlight their importance, and to emphasize the need to report bioassay results in IU while maintaining stocks of the international standard. Appendices had been included to provide practical guidance on general issues (such as the documentation and collaborative studies required for the development of secondary standards), along with specific procedural examples of the calibration of SARS-CoV-2, human papillomavirus and respiratory syncytial virus bioassays. Although the

manual had generally been well received by reviewers, a number of specific issues had been raised and were presented to the Committee for its consideration. In particular, the view of the Committee was sought on how to address the issue that different assay formats (such as neutralization and antibody binding assays) detect antibodies specific to different analytes, thus making it impossible to calibrate all assays to report the same quantitative value, and how best to resolve the associated suggestion that a SARS-CoV-2 antibody standard should only be used to calibrate secondary standards for use in neutralizing antibody assays.

Recognizing that the manual was intended to be broadly applicable to any secondary antibody standard, and that several examples existed where vaccine potency had been assessed using antibody binding assays, the Committee agreed with the inclusion of cautionary text advising developers and users of secondary antibody standards to give careful consideration to the choice of assay used in the development of the standard, and to the subsequent recommendations regarding its use. The Committee then went on to review the overall document and made several further suggestions. These included providing clearer guidance on the importance of measuring uncertainty for secondary standards, and on how this information should be used. The Committee also proposed that a number of editorial changes be made to further clarify the purpose and scope of the document, re-order the contents and make the text more consistent with the format of similar documents published in the WHO Technical Report Series. It was further recommended by the Committee that workshops be organized for NCLs and manufacturers on implementing the guidance set out in the manual on the preparation of secondary standards.

Subject to the above changes being made, the Committee recommended that the document WHO/BS/2022.2415 be adopted and annexed to its report (Annex 2).

3.2 Biotherapeutics other than blood products

3.2.1 Guidelines on evaluation of biosimilars

The 2009 WHO Guidelines on evaluation of similar biotherapeutic products (SBPs) set out the scientific principles for the development and evaluation of biosimilars, and have served well as the basis of national regulatory frameworks for the licensure of such products. As a result, they have facilitated the development of, and worldwide access to, biological products of assured quality, safety and efficacy at more affordable prices. However, in light of World Health Assembly resolution WHA67.21 on access to biotherapeutics, and technological advances in the production and characterization of biotherapeutic products, the Committee at its meeting in October 2020 had recommended reviewing the current scientific evidence and increased experience gained in this area to inform revision of the 2009 WHO Guidelines. At its meeting in December 2020, the

Committee had been updated on progress and informed of potential revisions that would make the Guidelines more permissive without compromising basic principles. The Committee had indicated its support for the revision process and advised that any resulting document should offer greater flexibility and reduced regulatory burden, while continuing to ensure the quality, safety and efficacy of biosimilars.

The Committee was provided with an overview of the document preparation process during which drafting group members had provided extensive expertise and perspectives from various countries, supplemented by contributions from diverse organizations across the six WHO regions. The subsequent review process had included two rounds of public consultation during which positive and supportive feedback had been received. The Committee was also provided with a detailed overview of the document contents, along with a summary of the main differences between the current document and the 2009 Guidelines. Among these, the scope had now been expanded to include biological products other than biotherapeutics, which would allow for other well-characterized biological products (including prophylactic products and recombinant analogues of blood products) to be included – with a corresponding shift to the use of the terms "biosimilar" rather than "similar biotherapeutic product" and "reference product" rather than "reference biotherapeutic product". The sections on quality, and nonclinical and clinical evaluation had also been extensively revised to make them more consistent with current practices, and with other guidelines, as well as to provide more clarity and flexibility. Among the specific changes made was the provision of new guidance on determining the need for in vivo animal studies and on the implementation of the 3Rs principles ("Replace, Reduce, Refine") to minimize the use of animals in testing.

During discussion, the Committee was asked whether the 2018 *WHO Questions and Answers: similar biotherapeutic products* document should be discontinued in light of the revised Guidelines, and whether the development of product-specific guidelines or case studies would be helpful to users of the Guidelines. The Committee recommended that the 2018 document be withdrawn. Noting the likely difficulty of developing product-specific guidelines, the Committee advised that a product-specific case study – for example, on an insulin product – be developed to provide users of the Guidelines with clear step-by-step guidance on developing a biosimilar for licensure. Discussion then turned to which biological products might be considered for future inclusion in the scope of the Guidelines. The Committee noted that the principles for evaluating biosimilars could potentially be applied to chemically synthesized products (such as synthetic peptides) that are analogues of biologically manufactured products. There was agreement however that it was too early to consider vaccines for inclusion bearing in mind differences in their clinical evaluation compared to

other biological products, their use in large populations of healthy people rather than patients, and the need to first gain experience in applying the concepts in the revised Guidelines.

The Committee highlighted two areas where it felt that more information or guidance would be helpful, namely: (a) the need for a comprehensive description of what constituted a full dossier; and (b) the crucial need for a reliance system and associated criteria to be used when a non-local reference product was being considered for use. The Committee noted that relevant information on these two issues was already available in other WHO guidelines and these could be cited in the references section. The Committee also proposed that, in addition to the post-translational errors already mentioned in the Guidelines, mention should also be made of amino acid sequence variants which could occur through translational errors such as misincorporation during high-level expression. In addition, the Committee expressed concern that the use of international standards in the post-marketing phase was inadequately described and that such standards might therefore be inappropriately used, bearing in mind that the biosimilar life-cycle would diverge from that of the reference product.

The Committee then sought clarification of several terminology decisions made in the document, including clarification of the rationale behind removal of the term "stepwise approach" and whether the term "high similarity" had been sufficiently defined. Other terminology issues addressed included the apparent interchangeability of the terms "drug substance", "active drug substance" and "active substance". In addition, the Committee noted that the INN would be used in conjunction with the proprietary name in labelling, which would provide clarity in terms of distinguishing between the biosimilar and reference product. Furthermore, noting that the decision to accept a manufacturer's data was left to the NRA, the Committee raised the possibility that differences in interpretation or product acceptance would result in a lack of harmonization. Clarification was given that while this would allow for greater flexibility by the NRA, outcomes were still likely to be harmonized where the same principles were used for assessment. Finally, the relationship between immunogenicity and product efficacy was discussed, and clarification provided that the occurrence of immunogenicity (and of anti-drug antibodies) was not necessarily of concern as this often had no impact on the efficacy of the product and potential areas of concern in this regard had been addressed in the Guidelines.

The Committee expressed its agreement with the way in which the issues raised during public consultation had been addressed and welcomed the alignment of the revised WHO Guidelines with current practices among experienced regulators worldwide. After reviewing all of the proposed changes made during the current discussion, the Committee recommended that the document WHO/BS/2022.2413 be adopted and annexed to its report (Annex 3).

3.2.2 Guidelines for the production and quality control of monoclonal antibodies and related products intended for medicinal use

Over the past 25 years, therapeutic mAb products have become increasingly important for the treatment of a wide range of diseases in areas such as haematology, immunology and oncology. Numerous mAb products were now in clinical development and the global market for them was likely to continue growing at an increasing rate. At the same time, it is widely recognized that access to mAb-based products has largely been restricted to wealthier countries, due at least in part to limited experience of the regulation of such products in low- and middle-income countries and a lack of regulatory harmonization globally. Although the majority of approved therapeutic mAbs have been developed for the treatment of noncommunicable diseases, their short development time, rapid impact and good safety characteristics also make them highly suitable for use during public health emergencies such as the COVID-19 pandemic.

The Committee was informed that existing WHO guidance applicable to mAbs focuses on products targeting noncommunicable diseases and offers little advice on the development and evaluation of mAbs used to treat infectious diseases. In addition, since the adoption of the 1991 WHO Guidelines for assuring the quality of monoclonal antibodies for use in humans, extensive technological advances in mAb manufacture and quality assurance (most notably involving the use of recombinant DNA and cloning technologies) have largely rendered such early guidance obsolete. Although a number of other WHO guidelines are relevant to mAbs, these are general in nature. In October 2020, mindful of the accelerated development of mAb products for the treatment of COVID-19 and other infectious diseases, the Committee had endorsed a proposal to prepare new WHO guidance in this area. Following a review of existing WHO guidelines relevant to mAbs, the Committee had further agreed that the new guidance would consist of two documents – the first of which would address the manufacturing and quality control of mAbs and related products, regardless of therapeutic application or biosimilarity, while the second would provide guidance on the nonclinical and clinical evaluation of mAbs, supplemented if required by disease-specific guidance.

The Committee was provided with a detailed overview of the development, scope and content of the first of these documents – the WHO Guidelines for the production and quality control of monoclonal antibodies and related products intended for medicinal use – which was intended to replace the 1991 Guidelines and to be applicable to current and potentially future mAb manufacturing approaches and expression systems, including plant-based expression systems. Developed through an extensive international consultation process, which had included two rounds of public consultation, the Guidelines broadly followed the format of similar WHO guidance on the manufacturing and quality control of

biological products. Efforts had also been made to align the document with other internationally recognized guidelines.

The Committee welcomed the development of the document, noting its importance in the context of the ongoing COVID-19 pandemic and its broader applicability to therapeutic mAbs for both communicable and noncommunicable diseases. Having addressed a number of the specific comments arising from the second public consultation, the Committee went on to review the overall document and made several further suggestions, including the addition of an appendix listing the more commonly used expression systems. In addition, while acknowledging that mAbs expressed by messenger RNA were beyond the scope of the current document, the Committee felt that some aspects of the recently adopted WHO guidance document on regulatory considerations in evaluating the quality, safety and efficacy of messenger RNA vaccines may be applicable, as the manufacturing steps were likely to be similar. It was also noted that the prospective WHO Guidelines on the nonclinical and clinical evaluation of mAb products would likely offer some guidance on the evaluation of mAbs expressed by messenger RNA. In light of this, it was agreed that the text of the current document would be modified accordingly. The Committee further noted that the principal plant expression systems currently used for the production of mAbs were based on tobacco plants and as such were largely supported and/or funded by the tobacco industry. Therefore, the use of such expression systems would conflict with current WHO policies on tobacco products, including the acquisition policies of the WHO Prequalification programme.

After due consideration of the issues raised, the Committee recommended that the document WHO/BS/2022.2414 be adopted and annexed to its report (Annex 4).

4. International reference materials – cell and gene therapy products

4.1 Proposed new projects and updates – cell and gene therapy products

4.1.1 Proposed changes to the First WHO International Reference Panel for lentiviral vector integration copy number

Gene therapy based on integrating lentiviral vectors (LVs) is increasingly being used to restore cell function in rare inherited diseases or to endow cells with capabilities such as the ability to kill cancer cells. For example, the advent of genetically modified T-cell therapies, such as chimeric antigen receptor T-cell therapy, to treat cancer has led to a significant increase in the number of patients treated using this platform technology. To ensure both product efficacy and safety, regulators generally require that minimum and maximum gene copy numbers respectively are specified.

In 2016, the Committee had endorsed a project to develop a WHO international reference standard for lentiviral vector integration copy number quantitation, which was subsequently established in 2019 as the First WHO International Reference Panel for lentiviral vector integration copy number (NIBSC code 19/158). The panel comprised three separate international reference reagents with assigned values of 0 LV copies/cell (NIBSC code 18/142), 1.42 LV copies/cell (NIBSC code 18/126) and 8.76 LV copies/cell (NIBSC code 18/132). However, subsequent use of these international reference reagents revealed significant differences in the data obtained using quantitative polymerase chain reaction (qPCR) and droplet digital polymerase chain reaction (ddPCR) assays that could not readily be resolved and may be due to inherent variations in each of these technologies. In addition, potential users noted that removing the original reference to a house-keeping gene would provide for greater flexibility in the way in which cell numbers were derived. The Committee was therefore presented with a proposal to assign a value in LV copies/ampoule instead of LV copies/cell and to establish two separate WHO international reference reagents derived from material 19/158, each with different value assignments. The first of these (NIBSC code 19/158q) would be intended for qPCR applications and the second (NIBSC code 19/158d) for ddPCR. In addition, a single material containing no lentivirus would be provided as a diluent.

Commenting on the significant challenges associated with developing international reference materials for innovative and evolving technologies, the Committee broadly supported the proposed approach and felt that the prospective establishment of two WHO international reference reagents derived from material 19/158 and with different assigned values would be a good solution.

However, in the absence of a formal collaborative study report, it was not possible to recommend either the disestablishment of the existing WHO international reference panel or the establishment of the two proposed WHO international reference reagents in its place at the current meeting. The Committee requested that a report setting out the relevant data supporting this proposal be presented for consideration at its meeting in October 2022.

5. International reference materials – in vitro diagnostics

5.1 WHO international reference standards for in vitro diagnostics

5.1.1 WHO International Reference Reagent for anti-human neutrophil antigen-3a immunoglobulin G

Human neutrophil antigens (HNAs) are grouped into five allelic systems (HNA-1 to HNA-5). Alloantibodies to HNA have been implicated in neonatal alloimmune neutropenia, febrile nonhaemolytic transfusion reactions, transfusion-related alloimmune neutropenia, transfusion-related acute lung injury (TRALI) and severe early rejection in kidney transplants. Alloantibodies to HNA-3a (anti-HNA-3a) have frequently been associated with severe TRALI which is an important and under-reported cause of transfusion-related morbidity and mortality in many developed countries. Anti-HNA-3a detection is thus important in the investigation and diagnosis of HNA antibody-mediated conditions, and may also have a role to play in donor-screening programmes in certain circumstances. Currently, only one commercial assay kit is available and most clinical laboratories have developed their own in-house protocols based on published methods, with harmonization achieved to some degree by the International Society of Blood Transfusion (ISBT) Granulocyte Immunobiology Working Party. As the sensitivity of these methods is significantly affected by a wide range of protocol-variability and other factors, there is a need for a WHO international reference reagent for anti-HNA-3a to facilitate assay development and validation and to allow for the monitoring of operator, equipment and test performance.

A freeze-dried candidate material (NIBSC code 19/114) had been produced from a pool of plasma obtained from three blood donors with high anti-HNA-3a levels resulting from pregnancy. The candidate material had been evaluated for its suitability to serve as a minimum potency WHO international reference reagent for anti-HNA-3a immunoglobulin G (IgG) in an international collaborative study involving 15 laboratories in 12 countries using methods validated for clinical use. A minimum potency was assigned to the candidate material by determining the maximum (end-point) dilution at which a majority of study participants could still detect anti-HNA-3a IgG. Although most laboratories used a combination of different test methods with a wide range of end-point dilutions reported, only one out of 36 tests performed failed to detect anti-HNA-3a IgG in the candidate material at a 1 in 8 dilution. Additional testing for other anti-HNA or human leukocyte antigen antibodies (anti-HLA) indicated either none or weak-positive results that were lost at a 1 in 4 dilution of the candidate material. It was therefore proposed that a 1 in 8 dilution be assigned as the minimum potency dilution for the candidate material, which would also avoid ambiguities arising from the reporting of weak anti-HLA activity.

Accelerated degradation studies indicated no significant loss in potency after storage at elevated temperatures of up to 37 °C for 22 months, indicating that the candidate material would be stable during long-term storage at −20 °C and sufficiently stable to allow for shipment at ambient temperature.

The Committee emphasized that the most important indication for anti-HNA-3a testing was the diagnosis of HNA-antibody-mediated conditions. Although such testing could in theory also be used as part of donor-selection criteria for TRALI risk, there would likely be little practical benefit. For example, recent experience in the collection of COVID-19 convalescent plasma had indicated no shortage of donors or donations, and conducting additional testing may be difficult due to exigencies of time. Concern was expressed however regarding the lack of awareness of TRALI in many countries, which highlighted the importance of reinforcing communications efforts with other strategies such as haemovigilance. Including non-reporting countries in studies such as these, along with the provision of appropriate technical and financial support, would potentially strengthen TRALI awareness. In response to a query regarding the variable detection of anti-HNA-3b antibodies in the candidate material by laboratories using the same commercial kit, it was clarified that a number of factors, such as the cut-off levels used, could have affected the results.

The Committee considered the report of the study (WHO/BS/2022.2417), noted that it had received the endorsement of the ISBT Granulocyte Immunobiology Working Party, and recommended that the candidate material 19/114 be established as the WHO Reference Reagent for anti-human neutrophil antigen-3a immunoglobulin G with an assigned minimum potency of a 1 in 8 dilution.

5.1.2 First WHO International Standard for Lassa virus RNA for NAT-based assays; and First WHO International Reference Panel for Lassa virus RNA for NAT-based assays

Lassa virus (LASV) is a zoonotic virus transmitted to humans through infected rats or person-to-person through contact with infected bodily fluids. Although approximately 80% of infected people are asymptomatic, around 20% of infections result in severe disease, including viral haemorrhagic fever. The disease is endemic in several West African countries and its incidence has been increasing in recent years, with current estimates in excess of 300 000 cases per year. There is high genetic diversity among circulating strains, particularly within the small segment of LASV RNA, with seven lineages currently defined. Nucleic acid amplification technique (NAT)-based assays are valuable diagnostic tools during the acute phase when rapid early diagnosis is crucial, with such assays usually based on in-house published methods. However, the genetic diversity of LASV presents a challenge in the development of pan-lineage NAT-based assays,

with difficulties in sample access also an issue due to the designation of LASV as a hazard group 4 pathogen and its corresponding inclusion in bioterrorism agent listings in some countries. Nevertheless, the designation of LASV as a top-ten priority pathogen with outbreak potential by the WHO Blueprint for Research and Development: Responding to Public Health Emergencies of International Concern (R&D Blueprint) highlights the need to support diagnostics development. The availability of a WHO international standard would allow for comparable evaluation of NAT-based assay analytical sensitivity and limits of detection, while a WHO international reference panel comprising representative isolates of selected LASV lineages would support assay development and allow for laboratory proficiency assessments.

In collaboration with the Foundation for Innovative New Diagnostics (FIND) an international collaborative study involving 18 laboratories in 14 countries had been conducted to assess the suitability of two LASV candidate materials and a candidate reference panel to serve as a WHO international standard and WHO international reference panel for LASV RNA respectively. One of the candidate international standards (NIBSC code 21/112) had been prepared using a whole acid-heat inactivated Lineage IV Josiah strain virus isolate. The other candidate international standard (NIBSC code 21/110) was also based on the Lineage IV Josiah strain but had been prepared from an equimolar mix of two chimeric lentiviral particles containing either the small or large segment of LASV RNA. This synthetic method had previously been used to produce Ebola virus and SARS-CoV-2 non-replicating non-infectious materials suitable for use when access to the live virus was restricted. The candidate international reference panel had also been assembled using chimeric lentiviral particles produced in a similar manner and comprised representative LASV strains from Lineage II (NIBSC code 21/102), Lineage III (NIBSC code 21/106), Lineage V (NIBSC code 21/108) and Lineage VII (NIBSC code 21/104).

Results were obtained using a range of in-house and commercial quantitative and qualitative assays based on block, real-time and digital PCR technologies. Both of the Lineage IV candidate international standards were detected across all assays and demonstrated comparable levels of harmonization, reducing both inter-laboratory variation and data spread across Lineage II, IV and V samples, and showing good agreement between small and large segment target assays. However, for Lineage III and VII samples, expressing data relative to the candidate international standards resulted in a separation in potency estimates, with assay harmonization only evident when considering each candidate material individually. Measures of inter-laboratory variation using low potency dilutions of the candidate international standards indicated better levels of harmonization when potencies were expressed relative to the candidate material of a similar type. Given that the inactivated virus preparation was more representative of

the virus in a clinical sample, it was proposed that candidate material 21/112 be established as the WHO international standard. In contrast to the candidate international standards, variable detection was observed for candidate reference panel members, with the Lineage III and VII samples returning the highest number of false-negative results. A higher number of false-negative results were also obtained with assays targeting the small segment, reflecting the high genetic diversity between lineages.

Accelerated degradation studies indicated that both candidate international standards were very stable and could be shipped at ambient temperature. Using the Arrhenius equation, the predicted loss of potency for candidate material 21/110 was estimated at 0.001% per year when stored at −20 °C. There was insufficient degradation in candidate material 21/112 to estimate potency loss and this would be reassessed in May 2022. Stability testing of the candidate reference panel had also been undertaken to study the impact of slightly high residual moisture levels in candidate materials 21/102 and 21/104. Microbiological testing had also detected contamination with mould or yeast in candidate materials 21/104 and 21/108 which could also affect product stability.

Having ascertained that the available batch of 2100 ampoules would be sufficient to last around 5 years with careful curation, the Committee considered the report of the study (WHO/BS/2022.2419) and recommended that the candidate material 21/112 be established as the First WHO International Standard for Lassa virus RNA for NAT-based assays with an assigned potency of 4.0 $\log_{10}$ IU/ampoule. Noting the need to further evaluate the Lineage III and VII samples and to assess the impact of residual moisture content and microbiological contamination, the Committee further recommended that the establishment of the First WHO International Reference Panel for Lassa virus RNA for NAT-based assays be delayed to enable sufficient performance and stability data to be collected.

5.1.3 First WHO International Standard for anti-β2GPI immunoglobulin G

The detection of IgG autoantibodies against β2 glycoprotein I (anti-β2GPI) is the basis for diagnosing antiphospholipid syndrome (APS). Patients with APS are at higher risk of arterial/deep vein thrombosis, thrombosis in the brain and miscarriage. Although a number of commercial diagnostic test kits are available for the measurement of anti-β2GPI IgG, these have been associated with unacceptable levels of variability. A range of problematic issues have been identified, resulting mainly from differences in the immunometric methods and calibration procedures used, and the lack of a suitable reference material. In response to a request from the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC), the European Commission Joint Research Centre (JRC) and NIBSC had therefore initiated a project to develop a WHO

international standard for the calibration and/or quality control of immunoassays used to measure anti-β2GPI IgG in human serum.

A lyophilized candidate material (NIBSC code 21/266) had been produced using pooled defibrinated plasma collected from two donors diagnosed with APS mixed with normal human serum. An international collaborative study involving eight laboratories in two countries had been conducted to assess the candidate material for its suitability to serve as a WHO international standard and to assign a unitage. Results from a total of nine immunoassays were returned, with all methods based on traditional enzyme-linked immunosorbent assays (ELISAs) except for one automated chemiluminescence assay platform. Seven of the assays (including the automated platform) were semi-quantitative and two were quantitative. For almost all laboratories (n = 6), acceptable dilutional linearity and conformance to preset acceptance criteria were observed, indicating the suitability of the candidate material 21/266. A geometric mean value of 221.905 was calculated, with an assigned value of 200 IU/vial for the candidate material considered to be appropriate for the assays used. Commutability was assessed using eight immunoassays and 39 routine clinical samples obtained from patients with APS. The results submitted by three participants were excluded either because of technical error during measurement (n = 1) or failure to meet the acceptance criteria (n = 2). The remaining results indicated that the candidate material had the same inter-method properties as most of the routine clinical samples, with its use reducing the variability of the results obtained. Short- and long-term stability studies indicated that the candidate material 21/266 remained stable when stored at or below −20 °C, and should preferably be shipped in dry ice. Following an accelerated degradation study conducted over 8 months of storage, predicted loss of concentration using the Arrhenius equation was estimated at 0.005% per month when stored at −20 °C, which would ensure stability for at least a decade at this temperature.

The Committee questioned whether the use of source material from only two donors was sufficiently representative, and was informed that assessing commutability with serum from additional donors included in the pool would have been impractical, as hundreds of samples would have been required. The Committee further asked whether there had been sufficient geographical representation among study participants, with only eight laboratories participating and results from six used for the analysis. The Committee was informed that it had been considered more important to involve as many test kit manufacturers as possible. Clarification was also provided that results from the two laboratories that had not met the acceptance criteria had been excluded for not meeting key study parameters. In response to an enquiry from the Committee as to why only 208 vials out of a total of 2860 vials produced had been allocated as the WHO international standard, it was explained that the international standard

to be curated by NIBSC would be reserved for the establishment of secondary reference materials and not be available to end users for calibrating their own assays. The remaining vials would be curated by the JRC for the calibration of working standards – a common practice for materials that were hard to obtain.

The Committee expressed concern that the International Society on Thrombosis and Haemostasis (ISTH) had not been approached to provide its feedback, as would usually be done when establishing or discussing issues related to blood coagulation. Such feedback can provide insight into the clinical utility of establishing a particular standard, which would in this case have been useful given that APS patients were often clinically managed by haematologists in hospital coagulation departments. However, following clarification that the original request for reference material had originated from the IFCC Committee for the Harmonization for Autoimmune Testing which comprises experts in autoimmune disease testing, and that the IFCC working group had not raised any issues with the proposal, it was accepted that a sufficient number of experts had been consulted. Nonetheless, the valuable feedback received from ISTH in the past was recognized and the Committee underscored the importance of maintaining strong relationships and good communications with ISTH and other scientific associations in ensuring support for the establishment of standards. However, as delays in the submission of standards proposals had occasionally occurred due to misalignment of meeting timelines in other organizations, it was recognized that an appropriate balance was necessary. It was therefore recommended that the ISTH and other relevant organizations should routinely be included in the call for comments when measurement standards reports are posted for public consultation. This would ensure that relevant experts and organizations are involved in the process of public consultation without affecting the timeliness of project submissions.

The Committee considered the report of the study (WHO/BS/2022.2420) and recommended that the candidate material 21/266 be established as the First WHO International Standard for anti-β2GPI immunoglobulin G with an assigned value of 200 IU/vial.

5.2 Proposed new projects and updates – in vitro diagnostics

5.2.1 Proposed WHO international reference reagents for anti-human leukocyte antigen antibodies

Flow cytometric crossmatch assays and Luminex-based assays are both well-established clinical diagnostic methods used in the transplantation field. The assays are performed prior to organ transplantation to detect anti-HLA antibodies that may be detrimental to organ performance, or to identify de novo alloantibody produced after transplantation. Within the European Union (EU) these assays are regulated by the European Federation for Immunogenetics (EFI). To support

assay validation, monitor trends and allow for the setting of acceptance criteria for sensitivity, anti-HLA run controls with different levels of alloreactivity have been developed and manufactured at NIBSC over the last 20 years, with approximately 500 vials distributed per year. Even though CE-marked[8] in vitro diagnostics (CE-IVDs) can only be used as research reagents outside the European Economic Area (EEA), around 17% of orders came from outside the EU in 2021 and there is continued interest from laboratories outside the EEA for these reagents to be made available in a form that would allow them to be used as controls in diagnostic assays. As most international regulators have adopted or aligned their requirements to the EU regulations set up by EFI, which specify the type of controls required in anti-HLA antibody assays, global access to such controls is important. Furthermore, in the absence of readily available commercial alternatives, laboratories with no access to the NIBSC run controls rely mainly on unstable in-house preparations based on leftover patient samples. There is therefore a need for the global standardization of these highly variable bioassays, which would be facilitated by converting the current NIBSC anti-HLA run controls to WHO international reference reagents.

The Committee was informed that the current run controls are well established as CE-IVDs, with a long track record of real-time stability and performance. Background data were also available from fitness-for-purpose studies. The high background negative control (NIBSC code 10/142) had been established in 2010 as a replacement control based on assessment by three laboratories in the United Kingdom. The low background negative control (NIBSC code 17/212) and strong positive control (NIBSC code 17/238) had been established in 2017 and 2019 respectively based on a study involving 20 laboratories in the United Kingdom and 61 international laboratories participating in an external quality assessment scheme. The weak positive control (NIBSC code 07/214) now required replacing and a collaborative study to evaluate a prospective replacement material (NIBSC code 21/378) was planned for April 2022. Real-time stability monitoring of all the materials had demonstrated high levels of stability when the freeze-dried products were stored at −20 °C and 4 °C.

It was proposed that the current anti-HLA run controls be converted to WHO international reference reagents by developing the replacement batch of weak positive control (NIBSC code 21/378) as a WHO international reference reagent and using historical data from fitness-for-purpose studies as collaborative study data to also establish CE-IVD preparations 10/142, 17/212 and 17/238 as WHO international reference reagents, supported by NIBSC in-house data. On this basis, a proposal to establish the materials as WHO international reference

[8] The letters "CE" appear on many products traded on the extended Single Market in the EEA and signify that such products have been assessed to meet high safety, health and environmental protection requirements.

reagents could be submitted to the Committee for its consideration in October 2022, subject to the approval of the change-control processes at NIBSC. However, if the existing data could not be used or were considered to be inadequate then efforts would have to be made to enlist additional participants and potentially to broaden global participation.

The Committee noted that this proposal departed from the normal process for the establishment of international reference materials, as set out the WHO Recommendations for the preparation, characterization and establishment of international and other biological reference standards. This process typically starts with the submission of a project proposal for endorsement by the Committee and is followed by a definitive international collaborative study to evaluate all candidate materials. Any decision to endorse the proposed change in approach could therefore set a precedent with implications for future projects. At the same time, it was accepted that there was currently an unmet medical need for internationally recognized controls in this area.

The Committee further noted that the WHO Recommendations for the preparation, characterization and establishment of international and other biological reference standards clearly stipulates the requirement for an international collaborative study with broad international participation and inclusion of all anticipated assay methods. However, the Committee also wondered if, in this particular case, more than 13 years of real-time data on the fitness-for-purpose and stability of the CE-marked reagents might be sufficient to be presented in lieu of a formal collaborative study report to support establishment of the proposed WHO international reference reagents, particularly given the medical need and amount of supporting real-time data available. It was noted that standards prepared from scarce materials had been established in the past on the basis of data from fewer than 10 laboratories. Furthermore, as these are CE-marked materials, any significant issues relating to their use as assay run controls would already have been identified and reported. The Committee also acknowledged the challenge of acquiring additional data in a retrospective collaborative study and of combining the results with existing real-time data.

After due consideration, the Committee agreed that the approach taken should adhere to the WHO Recommendations for the preparation, characterization and establishment of international and other biological reference standards, and recommended that steps be taken to reconcile the proposal and available data with this guidance. Subject to such reconciliation and the provision of a formal collaborative study report for its consideration in due course, the Committee endorsed the proposal (WHO/BS/2022.2421) to convert the three current and one replacement NIBSC anti-HLA run controls to WHO international reference reagents.

6. International reference materials – standards for use in high-throughput sequencing technologies

6.1 WHO international reference standards for use in high-throughput sequencing technologies

6.1.1 WHO international reference reagents for gut microbiome analysis

Disturbance of the human gut microbiome (dysbiosis) is associated with a range of diseases including inflammatory bowel disease and colorectal cancer. Therapies based on restoring the gut microbiome to a healthy state were increasingly being evaluated in clinical trials. Studying changes in the microbiome is the principal way in which the outcome of such therapies can be assessed and is largely based on HTS of DNA in extracted samples. However, currently used HTS methods introduce bias at different steps and the lack of effective standardization of HTS protocols and analysis is regarded as the single biggest barrier to translational research and product development in this field.

Noting the increasing demand for the standardization of innovative biological therapies, the Committee had endorsed a proposal made at its meeting in 2019 to develop WHO international reference reagents for gut microbiome analysis by HTS. Two candidate materials had been developed based on DNA obtained from 20 core bacterial species frequently found in the human gut microbiome. The first such candidate material (NIBSC code 20/302) consisted of DNA from the different strains in equal proportions (Gut-DNA-Mix) while the second (NIBSC code 20/304) also consisted of DNA from the different strains but in differing proportions (Gut-DNA-HiLo) to simulate the natural variability in microbiome composition and to allow for evaluation of the sensitivity and specificity of HTS pipelines. In addition, a reporting system had been developed based on four criteria: (a) sensitivity – defined as the number of species correctly identified; (b) diversity – defined as the total number of species detected; (c) similarity of the result to the known composition of the reagent; and (d) the relative abundance of false-positive results in the dataset.

An international collaborative study had been conducted involving 23 laboratories in 11 countries to evaluate the suitability of the candidate materials to serve as WHO international reference reagents, and to establish minimum quality criteria based on the reporting system. The majority of participants were from Europe and North America, and included academic institutions, NRAs and official medicines control laboratories, contract research organizations and the pharmaceutical industry. The study consisted of three modules conducted in two phases. In the first phase, participants processed the samples using either a recommended protocol or their own in-house protocol. Participants then had the option of using shotgun sequencing (Module 1), 16S amplicon sequencing

(Module 2) or both methods. Following sequencing, participants analysed their data using their own bioinformatics approach or methods provided by NIBSC in GitHub repositories to ensure consistency. The second phase (Module 3) was designed to assess bioinformatics bias independently of sequencing bias and involved participants re-analysing all of the raw sequence files obtained during the first phase using their own bioinformatics pipelines. Study results obtained using the candidate materials indicated substantial variability among current methodologies, highlighting the urgent need for appropriate standardization in this area. Use of the candidate materials and reporting criteria allowed for the identification of issues and limitations in the sequencing and bioinformatics analysis, including the misclassification of species, overestimation of diversity, low sensitivity of methods and limitations in current pipeline technologies. An accelerated degradation study had indicated that the candidate materials were stable, with their composition remaining unchanged for at least 6 months at a range of temperatures.

The Committee acknowledged the importance of this project given the rapid development of gut microbiome therapies worldwide and the need for standardization efforts to support the implementation of HTS technologies in this area. The Committee specifically requested that an update be provided at its next meeting on the relative abundance of organisms in the reagents when adjusted for 16S copy number. Discussion was also held on the likely degree of representativeness of the proposed reference reagents with regard to gut flora found in other geographical regions.

The Committee considered the report of the study (WHO/BS/2022.2416) and recommended that candidate materials 20/302 (Gut-DNA-Mix) and 20/304 (Gut-DNA-HiLo) be established as WHO international reference reagents for gut microbiome analysis with no assigned unitage.

7. International reference materials – standards for use in public health emergencies

7.1 Proposed new projects and updates – standards for use in public health emergencies

7.1.1 Update on the development of the First WHO International Standard for SARS-CoV-2 antigen

The rapid and accurate detection of infection with SARS-CoV-2 is essential for the clinical management of patients and the implementation of infection control procedures. Although NAT-based assays are both accurate and sensitive, they require specialized laboratory equipment operated by trained staff. Conversely, antigen-detecting rapid diagnostic tests (Ag-RDTs) provide a relatively simple way for individuals to test themselves using nasal or nasopharyngeal swabs. Since the start of the COVID-19 pandemic, numerous Ag-RDTs have been developed and commercialized, with most using the nucleocapsid protein as the target antigen, some using the spike protein and others a combination of the two.

In December 2020, the Committee had endorsed a proposal to develop a common WHO international reference standard to facilitate the assessment and comparison of Ag-RDTs and to calibrate secondary standards used in their quality control. Since then, a pilot study involving 17 laboratories had been conducted using Ag-RDTs with US Food and Drug Administration emergency use authorization (EUA) or with WHO EUL to determine the most suitable antigen preparation for use as a candidate WHO international standard. Participants tested six antigen preparations – four of which were whole virus suspensions (each inactivated by a different treatment), one a recombinant nucleocapsid protein and one a recombinant spike protein. Of the inactivated virus samples evaluated, the one inactivated with 0.01% formaldehyde had been detected with the greatest sensitivity. The reactivity of a panel of formaldehyde-treated SARS-CoV-2 VOCs had then been evaluated to inform the selection of samples for inclusion in a definitive international collaborative study. Results indicated that the reactivity of the selected Delta variant (B.1.617.2) was ten-fold lower than other VOCs in the panel. Further investigation revealed that this reduction was also observed using live virus, leading to speculation on whether this was a result of the way in which the virus had been grown or differences in the amino acid sequence of the nucleocapsid protein. A replacement Delta variant (AY.1) and Omicron variant (BA.1), both of which have similar levels of reactivity compared to other members of the VOC panel, would now be included in the definitive study. The study will evaluate 15 Ag-RDTs representing a range of technologies that have EUA or EUL, along with four analyser-based (laboratory instrument)

tests. It was anticipated that the collaborative study outcomes would be submitted to the Committee for it consideration in October 2022.

The Committee enquired as to possible reasons for the decreased reactivity of the Delta B.1.617.2 variant material. Although the cause had not been determined, it was suggested that this may have resulted from a frame-shift mutation of the viral sequence (R262fs) in approximately 5% of the study sample. However, no corresponding reduction in antigen reactivity had been observed in the Beta B.1.351 virus sample, which has the same frame-shift, and investigations were ongoing. Noting the slight delay in the project due to this issue, the Committee acknowledged the overall progress made and looked forward to reviewing the collaborative study report in due course.

7.1.2 Update on the development of the Second WHO International Standard for anti-SARS-CoV-2 immunoglobulin; and First WHO International Reference Panel for antibodies to SARS-CoV-2 variants of concern

The Committee was updated on the ongoing development of a replacement for the First WHO International Standard for anti-SARS-CoV-2 immunoglobulin which, following unprecedented demand, had been depleted by August 2021. Although intended for use in neutralizing antibody assays and assigned a unitage of 250 IU/ampoule for this purpose, the reference material had also been shown to reduce inter-laboratory variation when used in antigen-specific antibody binding assays. Recognizing the urgent need for such a standard during the ongoing COVID-19 pandemic, the Committee had therefore recommended that the material also be made available as a research reagent for the harmonization of antibody binding assays without an assigned IU but with suitable representative data from the collaborative study provided in the instructions for use (IFU).

In late 2020, following establishment of the First WHO International Standard for anti-SARS-CoV-2 immunoglobulin and associated First WHO International Reference Panel for anti-SARS-CoV-2 immunoglobulins, VOCs began to emerge carrying mutations that rendered them more transmissible. The impact of such variants on the effectiveness of vaccines and therapeutics now requires continual evaluation and in October 2021, the Committee had endorsed a proposal to develop a panel of reference sera specific for SARS-CoV-2 VOCs that could be expanded as new variants emerged. It was intended that such a panel would facilitate the development of serological assays needed to study the impact of new variants on the efficacy of existing biological products and help to address issues raised by the use of a single WHO international standard for different VOCs.

The Committee was informed that an international collaborative study was under way to assess the suitability of two candidate materials to serve as the Second WHO International Standard for anti-SARS-CoV-2 immunoglobulin,

with units to be assigned against the first international standard. The study was also providing an opportunity to characterize the candidate reference panel of antisera for VOCs in terms of their reactivity and specificity in different assay systems and against different SARS-CoV-2 isolates. Relative to the First WHO International Standard for anti-SARS-CoV-2 immunoglobulin, convalescent plasma from individuals infected with a VOC have a higher antibody response to the same virus and an even more marked difference may become evident when plasma samples from Omicron-infected individuals were evaluated in due course. No unitage would be proposed as the panel was not intended to be used for calibration and the IFU will make it clear that the panel is also not a diagnostic tool for identifying which VOC has infected a patient.

Preliminary study data analysis had been conducted and an overview was presented to the Committee. Further statistical analysis was under way to verify this preliminary analysis and to evaluate the ability of the candidate international standard materials to reduce inter-laboratory variation. It was intended that both the proposed Second WHO International Standard for anti-SARS-CoV-2 immunoglobulin and First WHO International Reference Panel for antibodies to SARS-CoV-2 variants of concern would be presented to the Committee for its consideration in October 2022.

Acknowledging the importance of these reference materials and the challenges inherent in their development, the Committee reflected on the implications of not replacing the current international standard with a like-for-like material, as a like-for-like material would potentially allow for a continued single unitage. However, in practice the emergence of VOCs meant that whichever candidate material was chosen, a specific value would have to be assigned for each variant. The Committee noted that although the plasma used to prepare one of the candidate materials had been sourced prior to the emergence of the Omicron variant, it may still be an effective standard due to the cross-reactivity of the antibodies represented in the plasma pool. The Committee also commented on the large number of different neutralization methods that had been developed for SARS-CoV-2 and suggested that it would be useful to compare their performance, as had been done during evaluation of the current international standard. With regard to antibody binding assays, the Committee was informed that many diagnostic kit producers had assigned BAU to their internal calibrators based on the parallel use of the current international standard material as an interim NIBSC research reagent, with several clinical trials also reporting results converted to BAU.

The Committee looked forward to reviewing the results of the full collaborative study at its meeting in October 2022.

8. International reference materials – vaccines and related substances

8.1 WHO international reference standards for vaccines and related substances

8.1.1 First WHO International Standard for anti-enterovirus D68 serum (human)

Enterovirus D68 (EV-D68) was first detected in 1962 among children with pneumonia and bronchiolitis in the USA. Subsequent sporadic cases then occurred until a large outbreak associated with severe respiratory symptoms and neurological complications was reported in the USA in 2014. This was followed by biennial upsurges in severe EV-D68 disease worldwide. As a result of its increasing prevalence, severe symptoms and association with acute flaccid myelitis, primarily in children, EV-D68 was identified as a re-emerging pathogen of public health concern. Although no vaccine is currently available, several are in development and an international reference material was now required to standardize the measurement of neutralizing antibodies against EV-D68 induced by vaccination or natural infection. At its meeting in 2018, the Committee had therefore endorsed a proposal to develop a First WHO International Standard for anti-enterovirus D68 serum (human).

Fifty two plasma samples donated by NHS Blood and Transplant in the United Kingdom had initially been assayed for EV-D68 neutralizing antibody titre – with 15 of these samples (negative for Coxsackievirus 16 but positive for EV-D68) defibrinated and pooled to produce two candidate materials (NIBSC codes 20/154 and 20/172). The candidate materials had been filled and lyophilized in glass ampoules and stored at –20 °C. An international collaborative study involving 15 laboratories in China and Europe had then been conducted to determine the neutralization titre of nine coded human serum samples (including the two candidate materials) against both the prototype Fermon isolate from 1962 and a 2016 isolate from the Netherlands belonging to sub-clade B3. Participating laboratories performed three independent assays using their own in-house neutralization method. Study results indicated that the high geometric coefficients of variation typically associated with such bioassays were reduced when data were expressed as relative potencies against the candidate materials. In addition low intra-assay variability and good intra-laboratory repeatability were also observed. The results indicate that both candidate materials 20/154 and 20/172 would be suitable to serve as an international reference material for measuring neutralization activity in human sera against a wide range of currently circulating EV-D68 strains. Real-time and accelerated degradation studies indicated that the candidate materials remained stable for

at least 1 year when stored at −20 °C, and also following reconstitution and freeze-thawing.

The Committee, noting that this had been a well-conducted and interesting collaborative study, asked if the source sera had been obtained from convalescent individuals. Clarification was given that it is impossible to collect convalescent sera as there was currently no active EV-D68 surveillance – however, the number of high-titre samples observed indicate that the virus is circulating widely. High-titre sera had been selected for use as candidate materials to try and ensure the specificity of the resulting reference material. Acknowledging that the candidate materials could be used with a wide range of EV-D68 isolates, including those from Africa, the Committee discussed the likelihood that their neutralization titres would vary when using virus isolates from different clades. The Committee concluded that the way in which the individual sera had been pooled meant they would not necessarily have the same titres with different clades, and it would be interesting to monitor the effects of diverse isolates as they emerged.

The Committee considered the report of the study (WHO/BS/2022.2418) and recommended that the candidate material 20/172 be established as the First WHO International Standard for anti-enterovirus D68 serum (human) with an assigned potency of 1000 IU/ampoule.

8.2 Proposed new projects and updates – vaccines and related substances

8.2.1 Proposed WHO international reference reagents for non-endotoxin pyrogens

Testing for the presence of pyrogenic substances (pyrogens) that could cause severe adverse effects in the recipients of vaccines and biotherapeutics is an important part of ensuring the safety of such products. Pyrogens include bacterial endotoxins along with a range of other microbial constituents. Testing for bacterial endotoxins is typically based on their reaction with Limulus amoebocyte lysate, an aqueous extract of blood cells obtained from the endangered horseshoe crab. To reduce dependency on an endangered animal species and ensure an adequate supply of reagents, recombinant Factor C has increasingly been used for endotoxin testing but this does not detect the presence of non-endotoxin pyrogens (NEPs) and so cannot be used when there is a risk that a biological product may contain such pyrogens.

Historically, the rabbit pyrogen test (RPT) has been used to detect pyrogens in biological products. However, this approach is not standardized, does not include a positive control, and its reproducibility is highly dependent on the source and husbandry of the rabbits. In addition, global concerns regarding animal welfare and the sustainability of such pyrogen testing have led

to considerable interest in the development and use of alternative approaches. As a result, over the last 25 years, the RPT has increasingly been replaced by the monocyte activation test (MAT) which measures the release of cytokines (for example, interleukin 1 beta, interleukin-6 or tumour necrosis factor alpha) from monocytes exposed to pyrogens, and which is standardized using the Third WHO International Standard for endotoxin. From 2026, the use of the RPT will be proscribed in Europe and international NEP reference materials for the qualification of cell batches used in the MAT are therefore now needed to support the further development and implementation of this assay and its global acceptance by regulators.

In 2015, the Committee had endorsed a proposal to develop a First WHO International Reference Reagent for Pam3 CSK4, a non-endotoxin toll-like receptor (TLR) ligand for use as a positive control in the MAT. However, the European Pharmacopoeia requires two such positive controls for validation purposes. A proposal was therefore being made to develop two further and complementary candidate NEP reference materials using a panel of recombinant and synthetic TLR agonists known to be free from endotoxin contamination. Once identified, the candidate materials will be filled and freeze-dried, and then evaluated in a collaborative study by qualified MAT users to assess their inter-laboratory reproducibility. The project will be collaboratively conducted by NIBSC and the Paul-Ehrlich-Institut (PEI).

Reflecting on its earlier discussions on strengthening the guidance provided on the 3Rs principles in WHO written standards (see section 2.1.3 above), the Committee agreed that the replacement of the RPT with the MAT was a high priority, and that progress in this respect would be supported by the development of further NEP reference materials. The Committee recognized the challenge presented by the diversity of NEPs and their relationship to different TLRs, some of which were not yet fully understood. The Committee endorsed the proposal (WHO/BS/2022.2421) to develop two WHO international reference reagents for NEPs for use in the MAT. Noting the importance of the cooperation between NIBSC and PEI in this project, the Committee also highlighted the need to reinstate the biennial meetings of the WHO collaborating centres as soon as was feasible.

8.2.2 Update on the development of WHO international standards for antibodies against human papillomavirus types 6, 11, 31, 33, 45, 52 and 58

Vaccines against human papillomavirus (HPV), the cause of cervical and some other cancers, have been licensed for more than a decade and since their introduction have been formulated to target an increasing number of HPV serotypes. The harmonization of HPV serological methods is crucial for

assessing the immunogenicity of vaccines and monitoring their performance in different populations. It is also important when measuring past or present HPV infection during epidemiological studies. WHO international standards for antibodies against HPV types 16 and 18 have previously been established, and the Committee was provided with an update on the development of proposed WHO international standards covering the remaining seven types found in commercially available vaccines.

Candidate materials derived from sera obtained from at least two individuals that had naturally been infected with the HPV type of interest – and who were preferably reactive to only one genital HPV type each (monospecific) – had been sourced from donors in China, Slovenia and Thailand. An international collaborative study had now been conducted involving 11 laboratories in seven countries to characterize the candidate materials for their reactivity and specificity in both multiplex antibody binding assays and pseudovirus neutralization assays. Consensus results indicate that the candidate materials for HPV types 6, 31, 33, 45, 52 and 58 are monospecific for their target antigen. However, the candidate material for HPV11 was not specific, and cross-reacted with HPV6, HPV33, HPV58 and others in certain assays. Nevertheless, the intention was still to pursue the establishment of this candidate material given its likely utility in harmonizing HPV11 assay results, and the view of the Committee with regard to this intention was invited.

Noting that laboratories currently rely on their own standardization approaches to harmonize their serological assays for HPV types, the Committee highlighted the ongoing need for global assay harmonization, which would be facilitated by the availability of international standards to allow for reporting in a common unitage. The Committee noted that the lack of monotypic specificity of the proposed HPV11 candidate material had been attributed to the presence of documented cross-reactive epitopes with other HPV types. However, given the current needs of the HPV field and the likely considerable difficulties in sourcing a more suitable HPV11 candidate material in the foreseeable future, the Committee agreed with the intention to pursue the establishment of the current candidate material. Reflecting on the complexity of the data presented in this update, the Committee indicated that the collaborative study report, which was expected to be presented at its meeting in October 2022, should clearly explain how such data should be interpreted.

Annex 1

WHO Recommendations, Guidelines and other documents related to the manufacture, quality control and evaluation of biological products

WHO Recommendations, Guidelines and other documents are intended to provide guidance to those responsible for the development and manufacture of biological products as well as to others who may have to decide upon appropriate methods of assay and control to ensure that such products are safe, reliable and potent. WHO Recommendations (previously called Requirements) and Guidelines are scientific and advisory in nature but may be adopted by an NRA as national requirements or used as the basis of such requirements.

Recommendations and guidance on biological products are formulated by international groups of experts and published in the WHO Technical Report Series[9] as listed below. A historical list of Requirements and other sets of Recommendations is available on request from the World Health Organization, 20 avenue Appia, 1211 Geneva 27, Switzerland.

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

WHO Press
World Health Organization
20 avenue Appia
1211 Geneva 27
Switzerland
Email: bookorders@who.int
Website: http://apps.who.int/bookorders

Individual Recommendations and Guidelines and other documents may be obtained free of charge as offprints by writing to:

Technical Standards and Specifications unit
Department of Health Product Policy and Standards
Access to Medicines and Health Products
World Health Organization
20 avenue Appia
1211 Geneva 27
Switzerland

[9] Abbreviated in the following pages to "TRS".

Recommendations, Guidelines and other documents	Reference
Animal cells, use of, as in vitro substrates for the production of biologicals	Revised 2010, TRS 978 (2013)
BCG vaccines (dried)	Revised 2011, TRS 979 (2013)
Biological products: good manufacturing practices	Revised 2015, TRS 999 (2016)
Biological standardization and control: a scientific review commissioned by the UK National Biological Standards Board (1997)	Unpublished document WHO/BLG/97.1
Biological substances: International Standards and Reference Reagents	Revised 2004, TRS 932 (2006)
Biosimilars, evaluation of	Revised 2022, TRS 1043 (2022)
Biotherapeutic products, changes to approved biotherapeutic products: procedures and data requirements	Adopted 2017, TRS 1011 (2018)
Biotherapeutic products, similar	Adopted 2009, TRS 977 (2013)
Biotherapeutic protein products prepared by recombinant DNA technology	Revised 2013, TRS 987 (2014); Addendum 2015, TRS 999 (2016)
Blood, blood components and plasma derivatives: collection, processing and quality control	Revised 1992, TRS 840 (1994)
Blood and blood components: management as essential medicines	Adopted 2016, TRS 1004 (2017)
Blood components and plasma: estimation of residual risk of HIV, HBV or HCV infections	Adopted 2016, TRS 1004 (2017)
Blood establishments: good manufacturing practices	Adopted 2010, TRS 961 (2011)
Blood plasma (human) for fractionation	Adopted 2005, TRS 941 (2007)
Blood plasma products (human): viral inactivation and removal procedures	Adopted 2001, TRS 924 (2004)
Blood regulatory systems, assessment criteria for national	Adopted 2011, TRS 979 (2013)
Cholera vaccines (inactivated, oral)	Adopted 2001, TRS 924 (2004)
Dengue tetravalent vaccines (live, attenuated)	Revised 2011, TRS 979 (2013)

Recommendations, Guidelines and other documents	Reference
Diphtheria, tetanus, pertussis (whole cell), and combined (DTwP) vaccines	Revised 2012, TRS 980 (2014)
Diphtheria vaccines (adsorbed)	Revised 2012, TRS 980 (2014)
DNA vaccines, plasmid	Revised 2020, TRS 1028 (2021)
Ebola vaccines	Adopted 2017, TRS 1011 (2018)
Enterovirus 71 vaccines (inactivated)	Adopted 2020, TRS 1030 (2021)
Haemophilus influenzae type b conjugate vaccines	Revised 1998, TRS 897 (2000)
Haemorrhagic fever with renal syndrome (HFRS) vaccines (inactivated)	Adopted 1993, TRS 848 (1994)
Hepatitis A vaccines (inactivated)	Adopted 1994, TRS 858 (1995)
Hepatitis B vaccines prepared from plasma	Revised 1994, TRS 858 (1996)
Hepatitis B vaccines (recombinant)	Revised 2010, TRS 978 (2013)
Hepatitis E vaccines (recombinant)	Adopted 2018, TRS 1016 (2019)
Human immunodeficiency virus rapid diagnostic tests for professional use and/or self-testing Technical Specifications Series for WHO Prequalification – Diagnostic Assessment	Adopted 2017, TRS 1011 (2018)
Human interferons prepared from lymphoblastoid cells	Adopted 1988, TRS 786 (1989)
Influenza vaccines (inactivated)	Revised 2003, TRS 927 (2005)
Influenza vaccines (inactivated): labelling information for use in pregnant women	Addendum 2016, TRS 1004 (2017) to Annex 3, TRS 927 (2005)
Influenza vaccines (live)	Revised 2009, TRS 977 (2013)
Influenza vaccines, human, pandemic: regulatory preparedness	Adopted 2007, TRS 963 (2011)
Influenza vaccines, human, pandemic: regulatory preparedness in non-vaccine-producing countries	Adopted 2016, TRS 1004 (2017)
Influenza vaccines, human, pandemic: safe development and production	Adopted 2018, TRS 1016 (2019)

Recommendations, Guidelines and other documents	Reference
In vitro diagnostics (WHO-prequalified), collaborative procedure between WHO and NRAs for assessment and accelerated national registration	Adopted 2020, TRS 1030 (2021)
In vitro diagnostic medical devices, establishing stability of, Technical Guidance Series for WHO Prequalification – Diagnostic Assessment	Adopted 2017, TRS 1011 (2018)
Japanese encephalitis vaccines (inactivated) for human use	Revised 2007, TRS 963 (2011)
Japanese encephalitis vaccines (live, attenuated) for human use	Revised 2012, TRS 980 (2014)
Louse-borne human typhus vaccines (live)	Adopted 1982, TRS 687 (1983)
Malaria vaccines (recombinant)	Adopted 2012, TRS 980 (2014)
Measles, mumps and rubella vaccines and combined vaccines (live)	Adopted 1992, TRS 840 (1994); Note 1993 TRS 848 (1994)
Meningococcal polysaccharide vaccines	Adopted 1975, TRS 594 (1976); Addendum 1980, TRS 658 (1981); Amendment 1999, TRS 904 (2002)
Meningococcal A conjugate vaccines	Adopted 2006, TRS 962 (2011)
Meningococcal C conjugate vaccines	Adopted 2001, TRS 924 (2004); Addendum (revised) 2007, TRS 963 (2011)
Monoclonal antibodies, production and quality control	Revised 2022, TRS 1043 (2022)
Monoclonal antibodies as similar biotherapeutic products	Adopted 2016, TRS 1004 (2017)
Papillomavirus vaccines (human, recombinant, virus-like particle)	Revised 2015, TRS 999 (2016)
Pertussis vaccines (acellular)	Revised 2011, TRS 979 (2013)
Pertussis vaccines (whole-cell)	Revised 2005, TRS 941 (2007)
Pharmaceutical products, storage and transport of time- and temperature-sensitive	Adopted 2010, TRS 961 (2011)
Pneumococcal conjugate vaccines	Revised 2009, TRS 977 (2013)

Recommendations, Guidelines and other documents	Reference
Poliomyelitis vaccines (inactivated)	Revised 2014, TRS 993 (2015); Amendment 2019, TRS 1024 (2020)
Poliomyelitis vaccines (oral)	Revised 2012, TRS 980 (2014)
Poliomyelitis vaccines: safe production and quality control	Revised 2018, TRS 1016 (2019) Amendment 2020, TRS 1028 (2021)
Quality assurance for biological products, guidelines for national authorities	Adopted 1991, TRS 822 (1992)
Rabies vaccines for human use (inactivated) produced in cell substrates and embryonated eggs	Revised 2005, TRS 941 (2007)
Reference materials, secondary: for NAT-based and antigen assays: calibration against WHO International Standards	Adopted 2016, TRS 1004 (2017)
Regulation and licensing of biological products in countries with newly developing regulatory authorities	Adopted 1994, TRS 858 (1995)
Regulatory risk evaluation on finding an adventitious agent in a marketed vaccine: scientific principles	Adopted 2014, TRS 993 (2015)
Respiratory syncytial virus vaccines	Adopted 2019, TRS 1024 (2020)
RNA vaccines, messenger, for prevention of infectious diseases	Adopted 2021, TRS 1039 (2022)
Rotavirus vaccines (live, attenuated, oral)	Adopted 2005, TRS 941 (2007)
Smallpox vaccines	Revised 2003, TRS 926 (2004)
Snake antivenom immunoglobulins	Revised 2016, TRS 1004 (2017)
Sterility of biological substances	Revised 1973, TRS 530 (1973); Amendment 1995, TRS 872 (1998)
Synthetic peptide vaccines	Adopted 1997, TRS 889 (1999)
Tetanus vaccines (adsorbed)	Revised 2012, TRS 980 (2014)
Thiomersal for vaccines: regulatory expectations for elimination, reduction or replacement	Adopted 2003, TRS 926 (2004)
Thromboplastins and plasma used to control oral anticoagulant therapy	Revised 2011, TRS 979 (2013)

Recommendations, Guidelines and other documents	Reference
Tick-borne encephalitis vaccines (inactivated)	Adopted 1997, TRS 889 (1999)
Transmissible spongiform encephalopathies in relation to biological and pharmaceutical products[10]	Revised 2005, WHO (2006)
Tuberculins	Revised 1985, TRS 745 (1987)
Typhoid vaccines, conjugated	Revised 2020, TRS 1030 (2021)
Typhoid vaccines (live, attenuated, Ty21a, oral)	Adopted 1983, TRS 700 (1984)
Typhoid vaccines, Vi polysaccharide	Adopted 1992, TRS 840 (1994)
Vaccines, changes to approved vaccines: procedures and data requirements	Adopted 2014, TRS 993 (2015)
Vaccines, clinical evaluation: regulatory expectations	Revised 2016, TRS 1004 (2017)
Vaccines, regulatory considerations: use of human challenge trials	Adopted 2016, TRS 1004 (2017)
Vaccines, lot release	Adopted 2010, TRS 978 (2013)
Vaccines, nonclinical evaluation	Adopted 2003, TRS 927 (2005)
Vaccines, nonclinical evaluation of vaccine adjuvants and adjuvanted vaccines	Adopted 2013, TRS 987 (2014)
Vaccines, prequalification procedure	Adopted 2010, TRS 978 (2013)
Vaccines, stability evaluation	Adopted 2006, TRS 962 (2011)
Vaccines, stability evaluation for use under extended controlled temperature conditions	Adopted 2015, TRS 999 (2016)
Varicella vaccines (live)	Revised 1993, TRS 848 (1994)
Yellow fever vaccines (live, attenuated)	Revised 2010, TRS 978 (2013) Amendment 2021, TRS 1039 (2022)
Yellow fever vaccines, laboratories approved by WHO for the production of	Revised 1995, TRS 872 (1998)
Yellow fever virus, production and testing of WHO primary seed lot 213-77 and reference batch 168-736	Adopted 1985, TRS 745 (1987)

[10] Available online at: https://apps.who.int/iris/handle/10665/68932

Annex 2

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

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

Abbreviations

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

1. Introduction

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

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

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

The coronavirus disease 2019 (COVID-19) pandemic has led to a major global effort to develop vaccines and therapeutics, including antibody-based therapeutics. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the etiological agent of COVID-19 and causes mild or asymptomatic infection in the majority of cases; however, around 10% of cases require medical intervention and a small proportion result in severe pneumonia and death. In 2020, the First WHO International Standard for anti-SARS-CoV-2 immunoglobulin was established to facilitate the development and harmonization of serological assays to a common unitage (*4*). These assays provide information on potential immune correlates of protection and are essential in supporting the clinical development of vaccines and therapeutics, as well as the seroepidemiological studies required to assess the impact of COVID-19. The assays broadly fall into two categories – virus neutralization assays and antibody binding assays such as enzyme-linked

immunosorbent assays (ELISAs) (see Appendix 5). Plaque or foci reduction neutralization tests (PRNTs or FRNTs respectively) and microneutralization assays (see Appendix 6) are widely regarded as the reference methods for measuring potentially protective antibodies against many viral diseases. Such assays involve the use of live virus, which in the case of SARS-CoV-2 requires laboratories at biosafety level 3 (BSL3). However, the use of pseudotyped viruses (PVs) in neutralization assays (see Appendix 7) has been shown to be a potential alternative, including systems based on lentiviral and varicella zoster virus PVs widely used for detecting neutralizing antibody to SARS-CoV-2 (*5*, *6*). In addition to these virus neutralization assays, other functional assays for anti-SARS-CoV-2 antibodies include, but are not limited to, assays that measure antibodies that block the viral receptor binding domain (RBD) from binding to the ACE-2 receptor and antibody-dependent cellular cytotoxicity assays.

Current human papillomavirus (HPV) vaccines are based on virus-like particles consisting of recombinant capsid proteins. The standardization of assays for HPV capsid antibody (see Appendix 9) has supported vaccine development and continues to underpin epidemiological studies. In recent years, WHO IS for HPV antibodies have been established for virus serotypes 16 and 18.

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

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

2. Purpose and scope

Antibody reference materials are used to minimize the inherent variability across different assays used to evaluate antibody responses, and to ensure uniformity in the designation of potency or activity to immune sera and potentially therapeutic antibody preparations. The term "secondary standard" as used in this document

includes all such reference materials developed by regional or national authorities, manufacturers and others and calibrated against the WHO IS. Such secondary standards are intended to provide greater quantities of calibrated material than would otherwise be available from the limited supply of the IS.

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

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

The document is intended for use by NCLs and other laboratories requiring reference materials for antibody-based disease assays, manufacturers of secondary standards, manufacturers of vaccines and antibody-based assays (including antibody-based IVDs) and providers of external quality assurance schemes. The document supplements the guidance provided in the WHO Recommendations for the preparation, characterization and establishment of international and other biological reference standards (*1*). Analogous guidance has been published by WHO on the preparation of secondary standards for vaccines (*2*) and for IVDs for infectious diseases based on nucleic acid or antigen detection (*3*).

3. Terminology

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

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

Analyte: the biological constituent being measured in the bioassay.

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

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

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

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

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

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

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

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

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

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

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

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

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

I**ndependent assays**: mutually exclusive test procedures.

International biological measurement standard: a **certified reference material** (referred to as a WHO IS) derived from a biological substance (that is, one that cannot be fully characterized by physicochemical means alone and is measured using a bioassay) and which enables the results of biological or immunological assays to be expressed in the same way worldwide.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Traceability: the metrological property of the result of a measurement or the value of a standard whereby it can be related to stated references, usually

national or international standards, through an unbroken chain of comparisons, all of which have stated uncertainties.

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

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

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

4. Using biological standards

The purpose of metrological traceability is to ensure that a measurement takes into account all uncertainties and is an accurate representation of the material being measured. Thus, the results of an assay should be expressed in terms of the values obtained at the highest level of the calibration hierarchy (*7*) – which in the physical sciences means obtaining values in the International System of Units (SI). However, it is difficult to assign a value unambiguously in SI units to the biological activity of a complex analyte such as an antibody or immune serum. Instead, arbitrary units are assigned to the biological activity of the material by measuring its potency relative to an established reference standard. The approach taken by WHO to measuring biological activity is to establish the highest order reference standard (the WHO IS) with a value assigned in IU. Other lower-order biological reference materials for a given analyte can then be related through a sequence of comparisons traceable to the IS.

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

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

Property	WHO IS	Secondary standard	Tertiary standard
Alternative names	Highest order international conventional calibrator	Regional or national reference material or standard	Working reagent or standard; internal assay reference reagent
Calibration	Evaluated in an international collaborative study involving laboratories worldwide, different assays and different types of laboratories (usually 15–30 participants)	Calibrated against the WHO IS	Calibrated against the secondary standard
Unitage	IU/mL	IU/mL	IU/mL
Traceability	N/A	Yes	Yes
Uncertainty of measurement	No	Yes (assay specific)	Yes (assay specific)
Commutability	Must be determined experimentally relative to clinical specimens	Should be determined experimentally relative to clinical specimens	Consideration should be given to experimentally determining relative to clinical specimens
Material	Should resemble, as closely and feasibly as possible, the analyte being measured – for example, for SARS-CoV-2 antibody standards, natural samples from SARS-CoV-2 recovered or vaccinated individuals	Should resemble, as closely as possible, the analyte to be measured. However, for assay-specific secondary standards, recombinant antibodies or animal serum may be used, with laboratories encouraged to address commutability	Should resemble, as closely as possible, the analyte to be measured. Biological material similar to the tested sample (such as recombinant antibodies or animal serum) may be used, with laboratories encouraged to address commutability

Table 1 *continued*

Property	WHO IS	Secondary standard	Tertiary standard
Typical final format of standard	Lyophilized	Lyophilized, liquid or dry tube specimen	Liquid or dry tube specimen
Usage	Calibration of secondary standards; initial validation of new assay/platform	Calibration of tertiary standards; working standards; run controls; and calibrators	Working standards; run controls; calibrators
Establishment of standard	International agreement through a WHO international collaborative study, proposal for adoption and subsequent establishment on the recommendation of the WHO Expert Committee on Biological Standardization	May be calibrated in several ways: 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 (assay-specific secondary standards)	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

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

In many cases, the IS will not yet have been established at the time of early clinical studies and antibody assays will need to be standardized using an antiserum working reagent. This is particularly likely to be the case during public health emergencies (such as the COVID-19 pandemic) when vaccine and therapeutic antibody development proceeds at pace before sufficient convalescent

serum is available to produce the IS. However, provided that sufficient working reagent is retained, the results of these early studies could be retrospectively converted into IU once the IS has been established.

5. Principles for preparing secondary standards for antibodies

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

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

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

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

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

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

- Traceability – the process by which the unitage of each analyte is assigned to the secondary standard relative to the IS is the "traceability path", and should be clearly defined.
- Uncertainty – any formal definition of a secondary standard in terms of a higher-order standard (such as the IS) must include handling of uncertainty. Where several methods have been used to calibrate a secondary standard, it may not be valid to make assumptions across the methods with regard to a single underlying true value or a probability distribution of values to estimate uncertainty. In such cases, uncertainty will be assay specific.
- Value-assignment methodology – the traceability path and uncertainty are only valid for the assay methodologies used to assign the value of the standard. For some IS, units are assigned for specific assays (for example, in virus neutralization or enzyme immunoassays). In such situations, it will be necessary to value-assign a secondary standard using a specific assay method.
  In principle, it should not be necessary to recalibrate existing secondary standards when the IS is replaced but the suitability of the replacement in this regard should be checked before it is established.
- Stability – the stability of a secondary standard is usually monitored in real time against the IS. Ideally this should be checked through ongoing monitoring of a suitable parameter appropriate for the assay used (such as neutralization titre).
- Commutability – commutability is the extent to which the reference material will be a suitable standard for all of the various types of samples being evaluated. When appropriate and feasible, commutability should be assessed as part of a collaborative study by including a panel of the different types of samples for which the standard will typically be used.

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

IS are likely to be lyophilized to ensure their stability for many years. In contrast, secondary standards are used as working standards and, therefore, need to be formulated so that they are stable throughout the period of their use. Ideally standards are sterile – however, materials of low bioburden may be acceptable

provided that this does not interfere with the assay, or affect standard stability or safety, and that the materials are kept under appropriate conditions to minimize potential bioburden.

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

6. Planning

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

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

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

In light of such considerations, the likely annual demand can then be determined, and an appropriate volume of bulk material sourced and number

of containers prepared. Planning should also take into account the number of containers that may be required for calibration and stability studies. Appendix 1 lists the documentation and records to be compiled during a standardization project.

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

7. Selection of candidate material

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

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

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

To ensure the safety of the standard, individual donations should be negative for known bloodborne virus markers (for example, of human

immunodeficiency viruses and hepatitis viruses) and, if necessary, the treatment of candidate material using an appropriate validated method to reduce the risk of viral contamination should be considered. For example, the risk of the presence of enveloped viruses may be reduced using a solvent-detergent treatment. Consideration should be given to the potential impact of such treatment on the characteristics of the material in the assay systems in which it will be used.

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

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

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

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

8. Processing of final container

8.1 Quality aspects

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

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

8.2 Nature of the secondary antibody standard

Antibody standards may be lyophilized, liquid or frozen liquid. They are generally lyophilized, as experience has shown this to be a consistently stable format that facilitates distribution. Although this is the preferred option, there may be circumstances in which the immunological characteristics of the standard would be affected by lyophilization or subsequent reconstitution of the material. If lyophilization is not possible or desirable, the distribution of frozen or liquid standards may be considered depending on the stability of the material. Stability should be determined by temperature-stressing studies. If the secondary standard needs to be shipped under refrigerated conditions (2–8 °C) or as a frozen liquid, the cold chain during transportation should be validated. Repeated freeze-thawing of frozen standards should be avoided because of the potential impact on the stability of the material. To avoid unnecessary freeze-thawing, the fill volume should be considered carefully and an aliquoting strategy employed if freeze-thawing is absolutely necessary. Freeze-thaws, if any, should be documented and it should be demonstrated that this does not affect the activity of the material.

8.3 Container format

The choice of container should be evaluated during pilot studies and shown not to affect the characteristics of the standard. Studies have shown that reference standards stored in vials with elastomeric closures (such as rubber stoppers) may exhibit inferior storage stability compared to those supplied in flame-sealed glass ampoules (the preferred container for an IS). Vials with elastomeric closures are,

however, more convenient and may be more suitable for secondary standards used in certain assay formats. The suitability of the rubber closures for the chosen storage conditions should be assessed as some formulations become brittle at low temperatures, compromising the integrity of the seal. Vials should be of good quality glass appropriate for pharmaceutical use. Plastic vials may be required in certain circumstances – for example, to meet biocontainment requirements – in which case they should also be of pharmaceutical quality.

8.4 Microbial bioburden

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

8.5 Accuracy/consistency of fill

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

8.6 Freeze-drying cycles

Lyophilization conditions should be based on the need to deliver stable standards of good and consistent quality. Sample formulations intended for lyophilization may be analysed by thermal analytical methods and/or freeze-drying microscopy to determine the critical transition temperature and therefore suitable freezing conditions for successful subsequent drying. Vacuum conditions should be

selected based on the vapour pressure of ice at the chosen shelf temperature and the optimum temperature for early freeze drying at sub-ambient temperature (primary drying) should be selected based upon conditions that avoid the temperature of the reference material rising above that of the critical transition temperature determined for the formulation. In later stages (secondary drying), the temperature is ramped up to ambient temperature or higher to yield a reference material with low residual moisture. At the end of drying the standard should be stoppered in either a vacuum or a dry gas environment that will prevent the ingress of any atmospheric moisture into the container on storage. Antibody standards are typically stored under a dry, inert atmosphere such as nitrogen.

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

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

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

9. Characterization

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

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

- Appearance – a freeze-dried standard should comprise a consistent, well-formed cake. Collapsed freeze-dried material is often associated with high residual moisture and poor stability. Any inconsistencies observed among individual containers should be investigated. The appearance of the reconstituted standard should be checked for consistency and the absence of particulate matter. Liquid and frozen standards should also be examined for their appearance and the absence of particulate matter.

- Moisture – low moisture content is critical for the long-term storage of freeze-dried standards. Ideally, for long-term storage stability, the moisture content of the standard should be < 1% – though higher levels of residual moisture may be acceptable for secondary standards provided that monitoring studies against a higher-order reference material indicate satisfactory stability.
- Potency – it should be demonstrated that the material in the container has retained its immunological activity for the assays in which it will be used. Where possible, the assays used should be based on WHO or compendial guidance (for example, European Pharmacopoeia or United States Pharmacopoeia). Other assays should be validated or qualified as appropriate.

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

Safety

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

10. Calibration against the International Standard

10.1 Principles of calibration

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

10.2 Collaborative study

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

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

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

performed in the participating laboratories, there should be an assay performed by all participants, with the SOP provided in advance by the WHO collaborating centre. Alternatively, the compliance of the participating laboratories with the relevant ISO standard may suffice.

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

10.3 Single laboratory calibration

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

11. Statistical analysis

The following sections are based on a common statistical method used for the calibration of reference materials – that is, parallel-line (or curve) analysis as described by WHO (*11*) and the European (*12*) and United States (*13*) pharmacopoeias. Another common method is based on demonstrating linearity of the primary and secondary reference materials in the assay system used (*8*). Any statistical method that has been demonstrated to be a reliable approach to the calibration of such materials can be applied. Appropriate software for the statistical analysis should be available for the evaluation of the data, and the statistical analysis should be performed by staff with expertise in this field. Examples of software used for such statistical analyses are provided in Appendix 4.

11.1 Statistical models

The calibration study data should be analysed using the relevant statistical model for the assay. The statistical validity of the fitted model should be assessed for each individual assay run. For the parallel-line and probit models, the linearity and parallelism of the logarithmic dose–response relationships between the IS

and secondary standard should be evaluated and shown to meet the system and sample suitability criteria before the potency of the candidate secondary standard relative to the IS can be calculated. Parallelism could be demonstrated by means of a significance test for non-parallelism (*12*) – though an equivalence approach for the difference or ratio of slopes may be preferred (that is, the confidence interval for the ratio of slopes must entirely lie between predefined equivalence margins). In addition, the precision with which potency has been estimated should be provided, usually in the form of a 95% confidence interval for the estimate.

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

11.2 Collaborative study calibration using multiple assays

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

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

11.3 Single laboratory calibration

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

11.4 Calculation of measurement uncertainty

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

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

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

When developing and assigning a value to any secondary reference standard, evaluating the likely impact of accumulated uncertainty, and determining

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

12. Stability

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

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

13. Monitoring stability in storage

Secondary antibody standards should be stored at an appropriate temperature, established by the stability studies conducted during its development. The temperature of the storage facility should be monitored and recorded routinely

(for example, by using an automated temperature monitoring system), and alternative storage arrangements should be available in case of breakdown.

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

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

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

14. Responsibilities of the custodian laboratory

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

- Storage of the secondary standard under appropriate conditions established during development.
- Distribution of the secondary standard when requested under appropriate conditions established during development.
- Maintenance of complete records on the project, covering:
  - the source of the bulk standard and its characterization, before and after filling;
  - collaborative study protocol, results, statistical analysis and report;
  - results of stability studies;
  - storage, inventory and dispatch of the reference standard;
  - number of ampoules/vials of standard established and distributed;
  - recipients of the standard in case any issues arise that would require all users to be informed.
- Documentation of feedback from users.

- Maintaining awareness of relevant assay developments and of how the standard is being used.
- Monitoring stability by requesting feedback on the use of the secondary standard that might provide ongoing evidence of the stability of the material.
- Publishing the results of the collaborative study.
- Providing advice and training on the use of the standard.

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

15. Instructions for use and labelling

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

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

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

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

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

16. Dispatch of standards

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

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

17. Secondary standard replacement

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

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

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

18. Authors and acknowledgments

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Appendices

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

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

Appendix 1

Preparation and calibration of national standard substances of biologics

I. Definition

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

II. Classification of national standard substances

National standard substances are divided into two classes:

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

III. Preparation and calibration of national standard substances

1. Laboratories and clean rooms used to prepare national standard substances of biologics shall comply with the requirements of good manufacturing practices (GMP) for pharmaceutical products and good laboratory practices (GLP).

2. The National Control Laboratory (NCL) is responsible for calibrating national biological standard substances.
3. Research and development of new national standard substances:

   – **Selection of source materials** – the nature of source materials for national standard substances shall be identical to that of the sample to be tested. Source materials shall not contain any interfering contaminants. Source materials shall be sufficient in quantity and of adequate stability and high specificity.
   – **Filling containers** – filling containers shall be neutral borosilicate glass. Heat sealing of the ampoule after the freeze-dried standard substance has been added will improve the stability of the standard substance.
   – **Formulation, filling, lyophilization and sealing of containers** – the formulation and dilution of standard substances shall be performed as required. Any necessary stabilizers or other materials shall not affect the activity, stability or assaying processes of the standard substance, and shall not volatilize during lyophilization. Substances qualified in control tests shall be dispensed accurately with a precision of ± 1%. Substances that need to be dried for preservation shall be sealed immediately after lyophilization. Residual moisture in the freeze-dried substances shall not exceed 3.0%. It is necessary to ensure consistency in terms of the potency and stability of the substance in each container during the course of filling, lyophilization and sealing.
   – **Test items** – test items shall be appropriate to the characteristics and intended purposes of the standard substances used, including at least, but not limited to, tests for filling precision, residual moisture, sterility, biological activity/potency and stability.
   – **Calibration** – ***collaborative calibration***: the development and calibration of standard substances to be established shall be conducted collaboratively in at least three experienced laboratories. The participants shall adopt the same protocols, and statistical analysis of the calibrated results shall be performed (the calibrated results necessitate at least five independent valid results). ***Confirmation of activity*** (potency unit or toxicity unit): activity is typically expressed as the mean value of the calibrated results obtained by participating laboratories. Data from the collaborative calibration shall be collected and analyzed

statistically by the NCL. Standard substances shall be assigned their activity value using appropriate statistical analysis methods and officially released following approval.

- **Stability studies** – accelerated stability tests shall be performed during the development step. Candidate substances shall be placed at various temperatures (–20 °C, 4 °C, 25 °C and 37 °C) for further testing of biological activity or content. The activity or content of established standard substances shall also be checked periodically.

4. Preparation and calibration of a substitute lot of standard substance:

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

IV. Approval of standard substances

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

V. Labels and package inserts

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

VI. Use, release and storage of standard substances

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

Appendix 2

Documentation to be compiled during a standardization project

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

Appendix 3

Collaborative study documentation

Model templates for Invitation, Response form and Collaborative study protocol

I: Model template: Invitation

Dear _____

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

We would ask you to:

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

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

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

Yours sincerely,

_____

II. Model template: Response form

Name of participant: _____
Address: _____
Telephone number: _____
Email: _____

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

* Delete as appropriate.

If able to participate, please provide:

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

Signed: _____
Name: _____
Date: _____

Please return to: _____
Email: _____

III: Model template: Collaborative study protocol

Background

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

Information on materials to be included in the study

International Standard
Candidate secondary standard
Any other samples

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

Assay methods

WHO/pharmacopoeial and/or methods in use in laboratory

Design of study

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

Results and data analysis

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

Timelines

Include deadlines for the return of results

Result sheet – showing title of study

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

Please return to: _____
Email: _____

Collaborative study report – outline of required contents

Introduction

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

Materials

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

Participants

List participants and their locations.

Study design and assay methods

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

Results

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

- the numbers of valid and invalid results;
- the grounds for any exclusion of outlier results (for example, non-parallelism or nonlinearity);

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

Discussion/conclusions

Proposed value assignment

Tables and figures

Appendix 4

Software for statistical analysis of bioassay data

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

WHO Bioassay Assist

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

Subject to WHO approval, the software is provided free upon request from:
Dr Dianliang Lei – leid@who.int
Norms and Standards for Biologicals
Technical Standards and Specifications Unit
Health Products Policy and Standards Department
Access to Medicines and Health Products Division
World Health Organization, Avenue Appia 20, CH-1211 Geneva 27, Switzerland.

CombiStats™

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

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

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

Other useful software includes:

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

Appendix 5

SOP of ELISA for SARS-CoV-2 antibodies[11]

Summary

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

Health and safety

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

Equipment and materials

- Flat-bottom NUNC maxisorp 96-Well Plates (Fisher Scientific cat. no. 44-2404-21, or equivalent);
- Phosphate buffered saline (1X) (Gibco cat. no. 10010-023, or equivalent);
- Tween 20 (Fisher Bioreagents cat. no. BP337-500, or equivalent);
- Milk powder (Marvel, or equivalent);
- TMB substrate (Neogen cat. no. 309175, or equivalent);
- 1N sulphuric acid H2SO4 (Sigma-Aldrich cat. no. 339741, or equivalent);
- Polypropylene sterile conical tubes: 15 mL and 50 mL;
- Sterile, serological pipettes: 5 mL, 10 mL and 25 mL;
- Micropipette tips: 10 µL, 20 µL, 200 µL, 500 µL and 1000 µL;
- Sterile reservoirs (Fisher Scientific cat. no. 07-200-127, or equivalent);

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

- Multichannel pipette(s): 200 µL;
- Wypalls;
- Pipetboy (Integra Biosciences), or equivalent;
- Class II biological safety cabinet;
- Ultra-low freezer (–80 °C);
- Refrigerator at 4 °C (± 1 °C);
- WellwashTM Versa Microplate Washer (Thermo Scientific) or equivalent; and
- FLUOstar® Omega Microplate Reader (BMG Labtech) or equivalent

Proteins

This protocol was set up using the following proteins:

- NIBSC/CFAR (kindly donated by Dr P. Cherepanov, The Francis Crick Institute, London, the United Kingdom) based on the original SARS-CoV-2 isolate MN908947 (https://www.ncbi.nlm.nih.gov/nuccore/MN908947);
- SARS-CoV-2 S1 (#100979);
- SARS-CoV-2 RBD (#100981);
- SARS-CoV-2 N (#100982); and
- SARS-CoV-2 trimeric spike (#101007) produced by C. Ball (NIBSC) using plasmid obtained from Dr B. Graham (NIH/NIAID, Bethesda, MD, the USA).

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

Antibodies

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

- Related secondary antibody: anti-rabbit HRP (Sino Biological, G33-62G-SGC) – use at 1 in 10 000.

Procedure

Day 1: Coating ELISA plates

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

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

Day 2: ELISA assay

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

NB plate layout shows samples tested in duplicate

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

11. Wash plate 3 times with PBS/0.05% Tween 20 (v/v).
12. Add 50 µL of anti-human IgG (Fab specific) horseradish peroxidase-conjugated secondary antibody diluted 1:3000 in PBS/0.05% Tween 20 (v/v) with 5% milk.
13. Incubate at room temperature for 1 hour, covered.
14. Wash plate 3 times with PBS/0.05% Tween 20 (v/v).
15. Add 50 µL TMB to all wells.
16. Allow to develop for 10 minutes.
17. Stop the reaction after 10 minutes by adding 50 µL of 2M H2SO4 to all wells.
18. Read at 450 nm absorbance on a plate reader immediately.

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

IgM

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

IgA

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

Appendix 6

Microneutralization assay for coronaviruses[12]

Purpose

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

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

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

Materials

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

Reagents

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

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

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

- Growth medium – Dulbecco's MEM (Sigma, cat. no. D6546) or equivalent, supplemented with **10% fetal calf serum**, 2mM L-Glutamine (Sigma, cat. no. G7513) or equivalent – for example, Glutamax (Invitrogen, cat. no. 35050-038) – and 1% penicillin/streptomycin (Invitrogen cat. no. 15140148).
- Dulbecco's MEM (Sigma, cat. no. D6546) or equivalent, supplemented with **4% fetal calf serum**, 2mM L-Glutamine (Sigma, cat. no. G7513) or equivalent – for example, Glutamax (Invitrogen, cat. no. 35050-038) – and 1% penicillin/streptomycin (Invitrogen, cat. no. 15140148).
- Trypsin/EDTA solution (Sigma, cat. no. T4049) or equivalent – for example, TrypLE Express (Invitrogen, cat. no. 12604-013).
- Dulbecco's MEM (Sigma, cat. no. D6546) or equivalent.
- Formaldehyde solution (Sigma, cat. no. 47673) prepared at 4% (v/v) in PBS-A (upon preparation, keep in fridge, for up to 2 months).
- Phosphate buffered saline (ThermoFisher, cat. no. 10010023).
- Washing buffer – PBS/0.05% (v/v) Tween 20 – alternatively prepare by adding Tween 20 (Sigma, cat. no. P1379) to PBS.
- 0.1% Triton-X100 (Sigma, cat. no. X100) diluted in PBS.
- Blocking buffer – washing buffer + 3% (w/v) Marvel milk powder.
- K-Blue aqueous TMB substrate (Neogen, cat. no. 331177).
- Stop solution – 2N H_2SO_4.

Antibodies – pathogen specific

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

Procedure

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

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

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

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

Day 1

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

If performed in a BSL3 laboratory:

1. Turn on the MSC and ensure appropriate checks are performed.
2. Seed VERO cells (CCL-81) at 2 x 10^4 cells per well in a 96-well flat-bottom plate to achieve confluent monolayers the next day.
3. Close the lids of the plates and place in a sealed container, spray with 70% IMS, remove outer gloves and take hands out of the MSC, re-glove and then remove sealed box from the MSC.
4. Place in a 37 ºC; 5% CO2 incubator overnight, opening the box vent for gas exchange.
5. Alternatively, if this step is performed in a BSL2 laboratory, the plates will need to be transported to the BSL3 laboratory on the day of infection in a sealed container.

Day 2

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

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

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

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

Dilution 1	12 µL sample + 108 µL MEM (1/10 – final will be 1/20)

Dilution 2	60 µL dil 1 + 60 µL MEM
Dilution 3	60 µL dil 2 + 60 µL MEM
Dilution 4	60 µL dil 3 + 60 µL MEM
Dilution 5	60 µL dil 4 + 60 µL MEM
Dilution 6	60 µL dil 5 + 60 µL MEM, discard 60 µL
Positive Control	60 µL MEM (virus only – no antibody)
Negative control	120 µL MEM (no virus – cells only)

Change tips between dilutions to avoid carryover.

7. Dilute the virus stock in medium without serum or antibiotics (for example, MEM or DMEM) to add 60 µL containing 100 TCID50/well (for example, for a viral stock of 2 x 10^5 $TCID_{50}$/mL – 100 ÷ 2 x 10^5 = 0.5 µL virus stock per well and 50 µL virus stock diluted in 5950 µL serum-free medium to add 60 µL containing 100 $TCID_{50}$ to 100 wells).
8. To each antibody dilution and positive control add 60 µL of diluted virus prepared as above.
9. Close the lid of the plate and place in a sealed container, spray with 70% IMS, remove outer gloves and take hands out of MSC, re-glove and then remove sealed box from the MSC.
10. Place in the incubator at 37 °C; 5% CO_2 for 1 hour.
11. Transfer plates with the virus/antibody dilutions and the plates seeded to the MSC within a sealed container.
12. Using a multichannel pipette, gently remove culture medium from the plates with seeded cells.
13. Transfer 100 µL of virus/antibody dilutions, and positive and negative controls, into each relevant well of the cell plate. An example of a potential layout is shown in Fig. 1.

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

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

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

Sample 1			Sample 2			Sample 3			Sample 4		
CELLS ONLY						VIRUS ONLY					

Day 3

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

1. Turn on the BSL3 MSC and ensure appropriate checks are performed and recorded on log sheet prior to use.
2. Retrieve plates within sealed container from the incubator and transport to the MSC.
3. Using a multichannel pipette, remove culture medium from the plates, and wash cells with 200 µL PBS.
4. Add 200 µL of 4% formaldehyde solution in PBS to each well.
5. Close the lid of the plate and place in a sealed container, spray with IMS 70%, remove outer gloves and take hands out of MSC, re-glove and then remove sealed box from MSC. Transport to the fridge (4 °C).
6. Incubate the plate for more than 16 hours.

Day 4

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

14. Add 100 µl of TMB substrate per well, and incubate for 5–15 minutes.
15. Stop the reaction with 100 µL of 2N H_2SO_4.
16. Wipe the outside of the plate with a Wypall soaked in 70% IMS; leave the lid inside the MSC before transporting the plate to the reader.
17. Read plates at OD450 nm.

Appendix 7

Neutralization assay using SARS-CoV-2 spike lentiviral pseudotyped virus[13]

Summary

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

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

Materials

Cell lines

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

Cell culture medium

HEK-293T clone 17 cells

- Gibco DMEM (1X) + GlutaMAX (ThermoFisher, cat. no. 61965-026);

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

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

Plasmids/recombinant virus

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

Reagents

- 0.25% trypsin-EDTA solution (Sigma-Aldrich, cat. no. T4049)
- Gibco Opti-MEM I (1X) (ThermoFisher, cat. no. 31985-047)
- Gibco DMEM (1X) phenol free (ThermoFisher, cat. no. 31053-028)
- FuGENE HD transfection reagent (Promega, cat. no. E2311)
- Bright-Glo® luciferase assay system (Promega, cat. no. E2620).

Consumables/equipment

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

Procedure

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

Day 1: Seed target cells in preparation for transfection

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

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

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

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

1. Following 24 hours incubation, remove medium from transfected target cells and detach from surface by incubation with 0.25% trypsin-EDTA or by following standard laboratory protocol for re-suspension of adherent cell lines.
2. Count cells and dilute with culture medium to 2 x 10^5 cells/mL.
3. Add 100 µL per well of a 96-well microplate to give 2 x 10^4 cells/well.
4. Incubate at 37 °C; 5% CO_2 for a minimum of 2 hours.

5. If frozen, retrieve test samples and thaw at ambient temperature. It is recommended to include a positive control sample with known neutralizing activity and appropriate negative control sample each time an assay is performed.
6. Calculate the amount of SARS-CoV-2 PV required; it is recommended to use an input of 150–300 $TCID_{50}$/well.
7. Retrieve from −80 °C storage the required number of aliquots of the SARS-CoV-2 PV and thaw at ambient temperature.
8. In a 96-well sterile U-bottom plate, prepare a dilution series of each test sample, and positive and negative control samples, within a final volume of 60 µL culture medium, taking into account the 1:2 dilution after the addition of PV at step 9. A threefold dilution series is recommended performed at least in triplicate. Each plate should also contain control wells of cells only and PV only.
   As per the example layout shown in Fig. 1, 81 µL culture medium is added into row A and 60 µL into all remaining wells. Next, 9 µL serum is added into each well of row A before performing a threefold serial dilution by carrying 30 µL across the rest of the dilution series rows B–G, discarding the final 30 µL.
9. Dilute SARS-CoV-2 PV in culture medium to add 60 µL containing 150–300 $TCID_{50}$ to each well of the dilution plate except the cell-only controls, to which 60 µL of culture medium only should be added to each well.
10. Incubate at 37 °C for 30–60 minutes.
11. Transfer 100 µL from each well of the dilution plate to the 96-well culture plate seeded with target cells as described in step 3 above (> 2 hours earlier).
12. Incubate at 37 °C; 5% CO_2 for 48 or 60 hours.

Day 5 or 6: Acquisition of results and data analysis

1. Following the 48 or 60 hours incubation period, prepare the Bright-Glo® reagent by reconstituting the Bright-Glo® substrate (brown glass bottle) with addition of the Bright-Glo® buffer (white bottle). Mix by inversion until the substrate is thoroughly dissolved. Aliquot and store the reconstituted reagent at between −77 °C and −83 °C for up to 1 year. Thaw the Bright-Glo® reagent at temperatures below 25 °C, equilibrate to room temperature and mix well before use.

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

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

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

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Appendix 8

Calibrating SARS-CoV-2 immunoassay internal assay reference reagents to international standards and/or secondary standards[14]

Purpose

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

Scope

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

Definitions

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

Procedure principles

Assign an International Unit per millilitre (IU/mL) or binding antibody unit per millilitre (BAU/mL) to an internal assay reference reagent that is used daily. The unit will be dependent upon the type of calibrator being used – an international

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

standard (primary standard) will have an assigned IU, while a secondary standard will have another unit assigned (such as arbitrary units, mg or index value) unless it has been calibrated to the international standard – in which case, the units of the secondary standard will be IU/mL.

Procedure

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

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

Data analysis

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

The calibrator is treated as the reference, and the potency value for the calibrator may be found in the respective Instructions for Use (IFU) document. For reference, Table 1 shows the unitages of the First WHO International Standard for anti-SARS-CoV-2 immunoglobulin (NIBSC code 20/136)[15] when reconstituted in accordance with the IFU.

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

Fig. 1
Plate map of a calibration set up

Day 1 Plate 1	1	2	3	4	5	6	7	8	9	10	11	12
	C_STD	C_STD	NEG	PC1	STD_C1	STD_C2	STD_C3	STD_T1	STD_T2	STD_T3	C_STD	C_STD
A	50	50	50	50	200	200	200	200	200	200	50	50
B	100	100	150	150	400	400	400	400	400	400	100	100
C	200	200	450	450	800	800	800	800	800	800	200	200
D	400	400	1350	1350	1600	1600	1600	1600	1600	1600	400	400
			No Sample	PC2								
E	800	800	50	150	3200	3200	3200	3200	3200	3200	800	800
F	1600	1600	150	450	6400	6400	6400	6400	6400	6400	1600	1600
G	3200	3200	450	1350	12800	12800	12800	12800	12800	12800	3200	3200
H	6400	6400	1350	4050	25600	25600	25600	25600	25600	25600	6400	6400

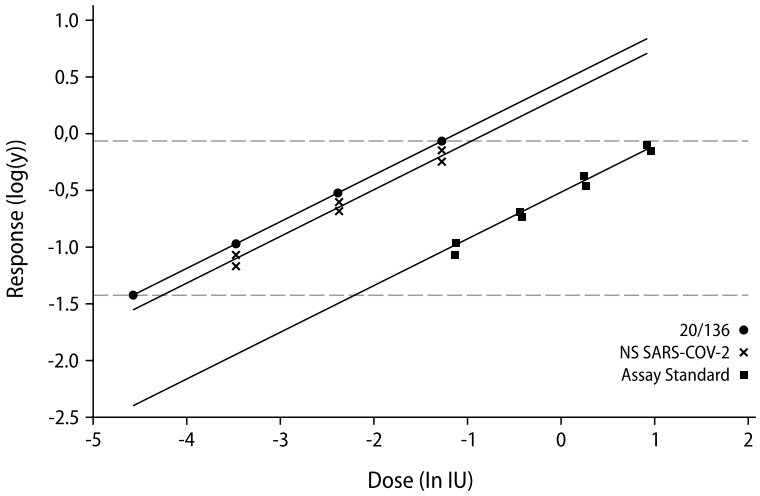
C_STD = internal assay reference reagent; STD-C1, C2 and C3 = calibrator; STD-T1, T2 and T3 = secondary standard; NEG = negative control; PC = positive control.

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

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

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

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



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

The estimated potency value calculated from the dose–response curve generated from each replicate series of serial dilutions for each sample is averaged for each day of testing. Finally, the geometric mean of the estimated potency values from each of the 3 days is calculated and will represent the final potency (calibrated) value for each sample. Table 2 illustrates the calibration process based on representative CombiStats™ data.

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

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

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

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

Calibration calculations

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

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

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

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

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

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

Calibration calculations

Assumptions:

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

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

Further reading

1. Recommendations for the preparation, characterization and establishment of international and other biological reference standards (revised 2004). In: WHO Expert Committee on Biological Standardization: fifty-fifth report. Geneva: World Health Organization; 2006: Annex 2 (WHO Technical Report Series, No. 932; https://www.who.int/publications/m/item/annex2-trs932?msclkid=69b33360c3e811ec9b73e5f303acf225, accessed 24 April 2022).
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Appendix 9

Calibrating human papillomavirus (HPV) immunoassay internal assay reference reagents to international standards and/or secondary standards[16]

Purpose

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

Scope

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

Definitions

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

Procedure principles

Assign an International Unit per millilitre (IU/mL) or binding antibody unit per millilitre (BAU/mL) to an internal assay reference reagent that is used daily. The unit will be dependent upon the type of calibrator being used – an international

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

standard (primary standard) will have an assigned IU, while a secondary standard will have another unit assigned (such as arbitrary units, mg or index value) unless it has been calibrated to the international standard – in which case, the units of the secondary standard will be IU/mL.

Procedure

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

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

Data analysis

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

Fig. 1

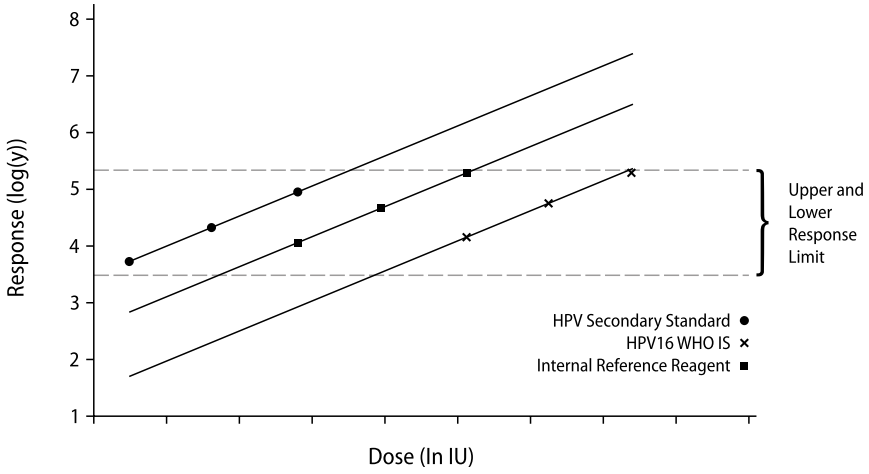
Plate map of a calibration set up

Day 1 Plate 1	1	2	3	4	5	6	7	8	9	10	11	12
	C_STD	C_STD	NEG	PC1	STD_C1	STD_C2	STD_C3	STD_T1	STD_T2	STD_T3	C_STD	C_STD
A	50	50	50	50	200	200	200	200	200	200	50	50
B	100	100	150	150	400	400	400	400	400	400	100	100
C	200	200	450	450	800	800	800	800	800	800	200	200
D	400	400	1350	1350	1600	1600	1600	1600	1600	1600	400	400
			No Sample	PC2								
E	800	800	50	150	3200	3200	3200	3200	3200	3200	800	800
F	1600	1600	150	450	6400	6400	6400	6400	6400	6400	1600	1600
G	3200	3200	450	1350	12800	12800	12800	12800	12800	12800	3200	3200
H	6400	6400	1350	4050	25600	25600	25600	25600	25600	25600	6400	6400

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

The calibrator is treated as the reference, and the potency value for the calibrator may be found in the respective Instructions for Use (IFU) document. Fig. 2 shows the dose (ln IU) versus response (log(y)) for: (a) the First WHO International Standard for antibodies to human papillomavirus type 16 (NIBSC code 05/134) (blue line); (b) a hypothetical HPV secondary standard (red line); and (c) a hypothetical internal assay reference reagent (green line). When reviewing such plots, it should be verified that the response values of the internal assay reference reagent and other reagents (such as the secondary standard) fall within the response range of the calibrator (see grey dotted lines in Fig. 2) to avoid performing analysis on extrapolated data. CombiStats™ allows the analyst to assign a relative potency value to the calibrator, and the programme will perform the relevant calculations needed to determine if the secondary standard and internal assay reference reagent are parallel to the calibrator – as depicted in Fig. 2. The analyst can then review the probability of the dose (ln IU) versus response (log(y)) lines being non-parallel and non-linear. A probability value greater than 0.05 for non-parallelism and non-linearity will indicate that the dose–response lines are parallel and linear. Furthermore, the CombiStats™ programme can calculate the relative potency of the samples, and this value in turn can be used to calculate the potency of the sample across the 3 days of testing.

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



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

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

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

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

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

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

Calibration calculations

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

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

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

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

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

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

Calibration calculations

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

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

Further reading

1. Recommendations for the preparation, characterization and establishment of international and other biological reference standards (revised 2004). In: WHO Expert Committee on Biological Standardization: fifty-fifth report. Geneva: World Health Organization; 2006: Annex 2 (WHO Technical Report Series, No. 932; https://www.who.int/publications/m/item/annex2-trs932?msclkid=69b33360c3e811ec9b73e5f303acf225, accessed 24 April 2022).

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

3. Gottschalk PD, Dunn JR. Measuring parallelism, linearity, and relative potency in bioassay and immunoassay data. J Biopharm Stats. 2005;15(3):437–63 (abstract: https://www.tandfonline.com/doi/abs/10.1081/BIP-200056532?journalCode=lbps20, accessed 24 April 2022).

4. Bates DM, Watts DG. Nonlinear regression analysis and its applications. New York: John Wiley & Sons, Inc.; 1988 (available from: https://onlinelibrary.wiley.com/doi/book/10.1002/9780470316757?msclkid=3a7e8630c3e911ecb48ce258f89aec9d, accessed 24 April 2022).

Appendix 10

Standardization of respiratory syncytial virus (RSV) neutralization assays[17]

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

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

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

Table 1
WHO IS for RSV

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

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

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

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

In line with the above, the WHO IS (NIBSC code 16/284) should be used when assaying neutralizing RSV antibody responses, and the results reported in IU along with information regarding the performance of the IS. This will allow

the scientific community to fully benefit from the new standard and improve understanding of neutralizing antibody responses. Feedback from users will also help WHO and NIBSC to promote the use of the IS and to develop other standards and reagents that may further improve the standardization of assays used in the clinical evaluation of RSV vaccines. Detailed information on the IS can be found at: http://www.nibsc.org/products/brm_product_catalogue/detail_page.aspx?catid=16/284

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

Table 2
BEI Resources RSV reference materials

BEI catalogue number	Wyeth lot number	BEI lot number	Material	Format
NR-4020	06594 (called "Reference" in Yang et al. (*4*)	V327-501-572	High-titre serum	1 mL (lyophilized)
NR-4021	06937 (called "Control I" in Yang et al. (*4*)	V327-512-572	Medium-titre serum	1 mL (lyophilized)
NR-4022	06938 (called "Control II" in Yang et al. (*4*)	V327-513-572	Medium-titre serum	1 mL (lyophilized)
NR-4023			Low-titre serum	1 mL (lyophilized)
NR-21973	CBER RSV Ig Lot 1	RSV-1	Purified Ig	2 mL (liquid)
NR-32832	Testing panel	63492188	Panel contains: • 1 vial NR-4020 (high-titre serum) • 1 vial NR-4021 (medium-titre serum) • 1 vial NR-4022 (medium-titre serum) • 1 vial NR-21973 (CBER RSV Ig Lot 1) • 1 vial: NR-49447 (Ig depleted serum)	Same respective formats as above

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

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

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

Box 1

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

Neutralization was measured using a previously reported fluorescence plate reader neutralization assay with modification. Sera were diluted in threefold serial dilutions from 1:10 to 1:65 610, mixed with an equal volume of recombinant mKate-RSV expressing prototypic F genes from subtype A (strain A2) or subtype B (strain 18537), and incubated at 37 °C for 1 hour. Next, 50 µL of each serum dilution/virus mixture was added to HEp-2 cells that had been seeded at a density of 2.4×10^4 in MEM (minimal essential medium) in each well of 384-well black optical bottom plates, and incubated for 23–24 hours before spectrophotometric analysis at 588 nm excitation and 635 nm emission (SpectraMax® M2e, Molecular Devices, CA). The IC50 for each sample was then calculated by curve fitting and non-linear regression using GraphPad Prism (GraphPad Software Inc., CA).

Box 1 *continued*

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

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

GMT = geometric mean titre

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

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

RSV neutralization assay method

Aim

To measure neutralizing antibody titres against RSV in serum samples

Assay outline

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

Reagents

- PBS
- DMEM media
- DMEM, high glucose, without sodium pyruvate

- penicillin/streptomycin (Pen/Strep)
- amphotericin B (AmpB)
- L-glutamine 200 mM
- methanol
- bovine serum albumin (BSA)
- fetal calf serum
- 30% hydrogen peroxide (H_2O_2)
- biotynylated anti-RSV antibody
- ExtrAvidin®–Peroxidase
- SigmaFast™ DAB substrate
- deionized water.

Solutions

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

Cell preparation

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

Neutralization assay

1. Heat-inactivate serum samples by incubating in a water bath for 30 minutes at 56 °C.
2. In a sterile 96-well plate, dilute serum samples as shown below in SF DMEM:
   - make up at least 150 µL of 1:10 dilution in a sterile Eppendorf;
   - take 120 µL of 1:10 dilution and add to column 1 of duplicate sample rows;

- add 60 µL of SF DMEM to columns 2 to 12 of sample rows;
- perform 2-fold serial dilution (60 µL added to 60 µL) from column 1 to 12 and discard final 60µL from column 12;
- add 60 µL of SF DMEM to Virus Only (VO) control wells; and
- add 60 µL of SF DMEM to Media Only (MO) control wells.

3. Defrost virus stock as quickly as possible then dilute to 2x required concentration.
4. Add 60 µL of 2x required virus concentration to sample wells and VO control wells. Add 60 µL SF DMEM to MO control wells. Cover the plate and incubate for 1 hour at 4 °C.
5. Remove HEp-2 cells from incubator and wash gently with 200 µL of SF DMEM.
6. Add 100 µL of sample/virus mix to the cells and allow adsorption for 2 hours at 37 °C; 5% CO_2.
7. After adsorption, add 100 µL of D4 and incubate for 24 hours at 37 °C; 5% CO_2.

Plate layout

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

Immunostaining

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

Calculating results

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

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

Positive well

Negative well

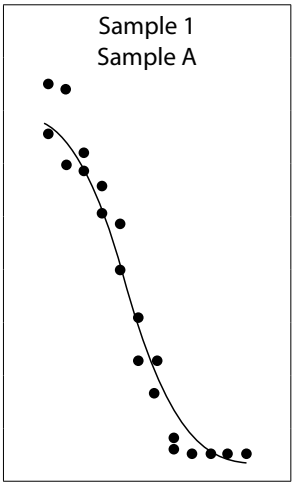
Exclude all wells without a complete monolayer from counting.

Example plate count

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

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

Example graph and results table



Sample 1			
Id.	Sample A		
(ul/vial)	Lower limit	Estimate	Upper limit
Potency	51.4056	63.4752	78.3611
Rel. to Ass.	51.4%	63.5%	78.4%
Rel. to Est.	81.0%	100%	123.5%
ED50/vial	865.541	1004.32	1165.60
Rel. to Ass.	865.5%	1004.3%	1165.6%
Rel. to Est.	86.2%	100%	116.1%

Conversion of ED_{50} titres to IU/mL

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

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

GMT = geometric mean titre

Example

Sample titre = 1200
WHO IS titre (ED50) = 1500
Calculation = 1200/(1500/2000) = 1200/0.75 = 1600
Neutralization titre of sample = 1600 IU/mL

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

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

Annex 3

Guidelines on evaluation of biosimilars

Replacement of Annex 2 of WHO Technical Report Series, No. 977

Guidelines published by the World Health Organization (WHO) are intended to be scientific and advisory in nature. Each of the following sections constitutes guidance for national regulatory authorities (NRAs) and for manufacturers of biological products. If an NRA so desires, these WHO Guidelines may be adopted as definitive national requirements, or modifications may be justified and made by the NRA. It is recommended that modifications to these Guidelines are made only on condition that such modifications ensure that the product is at least as safe and efficacious as that prepared in accordance with the guidance set out below.

Abbreviations

ADA	anti-drug antibody
ADCC	antibody-dependent cellular cytotoxicity
ADCP	antibody-dependent cellular phagocytosis
CDC	complement-dependent cytotoxicity
C1q	complement component 1q
Fab	antigen-binding fragment
Fc	fragment crystallizable
FIIa	activated blood coagulation factor II
FXa	activated blood coagulation factor X
G-CSF	granulocyte-colony stimulating factor
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
Ig	immunoglobulin
INN	international nonproprietary name
IS	international standard(s)
IU	International Unit(s)
mAb	monoclonal antibody
NRA	national regulatory authority
PD	pharmacodynamic(s)
PK	pharmacokinetic(s)
RP	reference product
SD	standard deviation
TNF	tumour necrosis factor

1. Introduction

Biotherapeutic products (biotherapeutics) have a successful record in treating many life-threatening and chronic diseases. The expiry of patents and/or data protection periods for a number of such biotherapeutics has ushered in an era of products that are designed to be highly "similar" to the corresponding licensed "originator" product. Based on a comprehensive head-to-head comparison and demonstrated high similarity, such products can partly rely for their licensing on safety and efficacy data obtained for the originator products. A variety of terms have been used to describe these products, including "biosimilars", "similar biotherapeutic products", "similar biological medicinal products" and "biosimilar products" (*1*).

The term "generic medicine" is usually used to describe chemical, small-molecule medicinal products that are structurally identical to an originator product whose patent and/or data protection period has expired. Demonstration of the analytical sameness and bioequivalence of the generic medicine to a reference product is usually appropriate and sufficient proof of therapeutic equivalence between the two. However, the approach established for generic medicines is not suitable for the development, evaluation and licensing of relatively large and complex proteins such as biosimilars.

As part of its mandate to assure the global quality, safety and efficacy of biotherapeutics, WHO provides globally accepted norms and standards for their evaluation. WHO written standards adopted on the recommendation of the WHO Expert Committee on Biological Standardization serve as a basis for setting national requirements for the production, quality control and overall regulation of biological medicines. In addition, WHO international measurement standards established by the Committee are essential for assessing the potency of biological medicines worldwide.

By 2007 an increasingly wide range of biosimilars were under development or were already licensed in many countries and a need for guidance on their evaluation and overall regulation was formally recognized by WHO (*2*). In 2009, the WHO Guidelines on evaluation of similar biotherapeutic products (SBPs) were adopted on the recommendation of the Committee (*3*). This document provided the scientific principles and stepwise approach to be applied during the demonstration of similarity between a similar biotherapeutic product and its reference biotherapeutic product. The document also provided guidance on the development and evaluation of such biotherapeutics; it was however viewed as a "living" document that would be further developed in line with advances in scientific knowledge and experience. It was also anticipated that the increasing availability of biosimilars worldwide would lead to increased competition between manufacturers, thus bringing down prices and improving access to such products.

In line with World Health Assembly resolution WHA67.21 on access to biotherapeutics (*4*), the Committee at its meeting in October 2020 recommended that a review should be undertaken of current scientific evidence and experience in this field to inform the updating and revision of the 2009 WHO Guidelines. This review would provide an opportunity to evaluate new developments and identify areas where the current guidance could be more flexible without compromising its basic principles, and allow for the provision of additional explanation of the possibility of tailoring the amount of data needed for regulatory approval (*5*). At its subsequent meeting in December 2020 the Committee was informed that the review had taken into account a range of national and regional guidelines, and a number of sections in the 2009 WHO Guidelines had been identified for potential updating and revision (*6*). Having been updated on progress in this area, the Committee expressed the opinion that the review of existing national and regional guidance had been comprehensive and indicated its support for the continuation of the proposed revision process (*5*). It was intended that the revision of the 2009 WHO Guidelines would result in greater flexibility and reduced regulatory burden, while continuing to ensure the quality, safety and efficacy of such products.

Following international consultations (*7*), the present document represents the outcome of the above revision process and replaces Annex 2 of WHO Technical Report Series, No. 977 (*3*). The main changes made include:

- Updating the Introduction to reflect the discussions held on the revision process.
- Expanding the scope of the document to include the evaluation of biological products other than biotherapeutics and a corresponding shift to the use of the term "biosimilar" rather than "similar biotherapeutic product".
- Use of the term "reference product (RP)" rather than "reference biotherapeutic product (RBP)" and updating of the considerations regarding the use of non-local RPs.
- Extensively revising the sections on quality, and nonclinical and clinical evaluation to make them more consistent with current practices, and with other guidelines, as well as to provide more clarity and flexibility – specific topics addressed include but are not limited to:
  - the use of WHO international standards and reference reagents;
  - analytical considerations in quality evaluation;
  - considerations in establishing similarity ranges for quality comparisons, and in determining similarity;

  - new guidance on determining the need for in vivo animal studies and on the implementation of the 3Rs principles ("Replace, Reduce, Refine") to minimize the use of animals in testing; and
  - consideration of the amount and type of clinical data required.

- Updating the sections on pharmacovigilance, prescribing information and label, and the role and responsibilities of national regulatory authorities (NRAs) with additional details and references.

For public health purposes, it is essential that the standard of evidence supporting the decision to license a biosimilar is sufficiently high to ensure that the product meets acceptable levels of quality, safety and efficacy. Elaboration of the data requirements and considerations for the licensing of such products is expected to facilitate the development of and worldwide access to biological products of assured quality, safety and efficacy at more affordable prices. It is expected that these WHO Guidelines on the scientific principles for evaluating biosimilars will help to harmonize global requirements, and lead to easier and speedier approval and assurance of the quality, safety and efficacy of these products. It is important to note that biological products that are not shown to be similar to an RP as set out in these Guidelines should not be described as "similar" and should not be termed "biosimilars".

It is recognized that a number of important issues associated with the use of biosimilars, including but not limited to the following, need to be defined by the individual NRA:

- intellectual property issues;
- interchangeability modalities, including switching (physician-led) and substitution (pharmacy-led) of an originator product with a biosimilar; and
- labelling and prescribing information.

For this reason, these issues are not elaborated upon in detail in this document.

2. Purpose and scope

These WHO Guidelines are intended to provide globally acceptable principles for the licensing of biological products that are claimed to be similar to biological products of assured quality, safety and efficacy that have been licensed based on a full licensing dossier. On the basis of proven similarity, the licensing of a biosimilar would in part rely on nonclinical and clinical data generated for an already licensed originator product. These Guidelines can be adopted by NRAs

worldwide or used as a basis for establishing national regulatory frameworks for the licensure of such biosimilars.

The Guidelines apply to biological products that can be well characterized, such as recombinant DNA-derived therapeutic peptides and proteins (*8*). Some of the principles provided in these Guidelines may also apply to low molecular weight heparins and recombinant analogues of plasma-derived products. Vaccines and plasma-derived products are excluded from the scope of these Guidelines.

3. Terminology

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

Biosimilar: a biological product that is shown to be highly similar in terms of its quality, safety and efficacy to an already licensed **reference product**.

Comparability/similarity exercise: direct head-to-head comparison of a biological product with a licensed **reference product** with the goal of establishing similarity in quality, safety and efficacy.

Comparability margin: the largest difference that can be judged as being clinically acceptable.

Comparability/similarity range: predefined allowable differences in physicochemical properties and biological activity level.

Drug product: a pharmaceutical product that typically consists of a **drug substance** formulated with **excipients**.

Drug substance: the active pharmaceutical ingredient and associated molecules that are typically formulated with excipients to produce the **drug product**. This may also be referred to as the "active substance" in other documents.

Efficacy study: a clinical trial to compare the efficacy of the biosimilar to the reference product.

Excipient: a constituent of a medicine other than the **drug substance**, added in the formulation for a specific purpose. While most excipients are considered inactive, some can have a known action or effect in certain circumstances (for example, hyaluronidase). The excipients may differ for a biosimilar and its reference product and need to be declared in the labelling and package leaflet of the medicine to ensure its safe use.

Equivalent: equal or highly similar in the parameter of interest. Equivalent quality, safety and efficacy of two medicinal products denotes that they can be expected to have similar (no better and no worse) quality, safety and efficacy, and that any observed differences are of no clinical relevance.

Generic medicine: a medicine that is structurally identical to an **originator product** (comparator) for which the patent and/or data protection period has expired.

Head-to-head comparison: direct comparison of the properties of a biosimilar with its corresponding **reference product**. Comparison based on historical data is not acceptable.

Immunogenicity: the ability of a substance to trigger an immune response or reaction (for example, development of specific antibodies, T-cell response, or allergic or anaphylactic reaction).

Impurity: any component present in the **drug substance** or **drug product** that is not the desired product, a product-related substance or **excipient** (including buffer components). Impurities may be either process or product related.

Marketing authorization holder: any person or legal entity that has received a marketing authorization or licence to manufacture and/or distribute a medicine. It also refers to a person or legal entity allowed to apply for a change to the marketing authorization or licence. Under the same licence, the marketing authorization holder could have several manufacturing sites registered. Therefore, several manufacturers could be involved.

Non-inferior: not clinically inferior to a comparator in the parameter studied. A non-inferiority clinical trial is one that has the primary objective of showing that the response to the investigational product is not clinically inferior to that of a comparator within a pre-specified margin.

Originator product: a medicine that has been licensed by an NRA on the basis of a full registration dossier – that is, the approved indication(s) for use were granted on the basis of full quality, efficacy and safety data.

Pharmacodynamic study: a clinical study that measures a pharmacodynamic (PD) response that effectively demonstrates the characteristics of the product's target effects. PD biomarkers for biosimilars do not need to be surrogate end-points for clinical efficacy outcomes.

Pharmacovigilance: the science and activities relating to the detection, assessment, understanding and prevention of adverse effects caused by medical drugs.

Posology: dosage for each indication and each method/route of administration. Information includes dose recommendation (for example, in mg, mg/kg or mg/m2), frequency of dosing (for example, once or twice daily, or every 6 hours) and treatment duration.

Reference product (RP): a biological product used as the comparator in a direct head-to-head **comparability exercise** with a **biosimilar** in order to demonstrate similarity in terms of quality, safety and efficacy. Only an **originator product** licensed on the basis of a full registration dossier and marketed for a suitable period of time with proven quality, safety and efficacy can serve as an RP.

Reference standard: a measurement standard such as an international, pharmacopoeial or national standard – it should be noted that reference standards are distinct from reference products and serve a different function.

Similarity: absence of any relevant difference in the parameter(s) of interest.

4. Scientific considerations and concept for licensing biosimilars

The regulatory framework for the licensing of generic medicines is well established in most countries. Demonstration of structural sameness and bioequivalence of the generic medicine and the reference product (RP) is usually sufficient for therapeutic equivalence between the generic and reference products to be inferred. However, the generic approach is not suitable for the licensing of biosimilars since biological products usually consist of relatively large and complex proteins that are more complicated to characterize and manufacture than small molecules.

Characterization and evaluation of the quality attributes of the RP should be the first step in guiding the development of the biosimilar. This is followed by a comparability exercise applying sensitive orthogonal analytical methods and assays to demonstrate structural, functional and clinical similarity. Comprehensive characterization and comparison showing similarity at the quality and nonclinical (in vitro) level are the basis for establishing comparability, with a tailored confirmatory clinical data package required for licensure. If differences between the biosimilar and the RP are found, the underlying reasons for them should be investigated. Unless such differences are explained and justified in terms of lack of clinical impact, additional data (for example, on safety) may be required. The standalone development of biological products is not discussed in the current Guidelines.

In addition to quality and nonclinical (in vitro) data, clinical data are generally required for any biosimilar. The type and amount of such data considered to be necessary will depend on the product or class of products, on the extent of characterization possible using state-of-the-art analytical methods, on observed or potential differences between the biosimilar and the RP, and on clinical experience with the RP (for example, safety/immunogenicity concerns in a specific indication). A case-by-case approach will be needed for each class of products.

A biosimilar is intended to be highly similar to a licensed biological product for which substantial evidence exists of its safety and efficacy. Manufacturers should demonstrate both a full understanding of their product and consistent and robust manufacture, and should submit a full quality dossier that includes a complete characterization of the product. Comparison of the biosimilar and the RP with respect to quality represents an additional element to the "traditional" full quality dossier. Such comparison will include a comprehensive comparison of biological function at the in vitro level. A reduction in data requirements is therefore possible for the nonclinical in vivo and/or clinical parts of the development programme. The posology and route of administration of the biosimilar should be the same as for the RP.

Studies must be comparative in nature and must employ state-of-the-art analytical methods capable of detecting potential differences between the biosimilar and the RP. The main clinical studies should use the final formulation of the biosimilar (that is, derived from the final process material); if not, then additional evidence will be required to demonstrate that the biosimilar to be marketed is comparable to that used in the main clinical studies (*9*).

If similarity between the biosimilar and the RP has been demonstrated, the biosimilar may be approved for all clinical indications of the RP supported by appropriate scientific data and justification (see section 9.7).

5. Key principles for the licensing of biosimilars

- Characterization of the quality attributes of the RP should be the first step in guiding the development of the biosimilar. The subsequent comparability exercise should demonstrate structural, functional and clinical similarity.
- Demonstration of similarity of a biosimilar to an RP in terms of structural and functional aspects is a prerequisite for establishing comparability, with a tailored clinical data package required as needed.
- A clinical bioequivalence trial with pharmacokinetic (PK) and pharmacodynamic (PD) parameters (if available), and including an assessment of immunogenicity in human subjects, will typically be a core part of the clinical comparability assessment, unless scientifically justified.
- The decision to license a biosimilar should be based on evaluation of the whole data package generated during the overall comparability exercise.
- If relevant differences between the proposed biosimilar and the RP are found at the structural, functional or clinical level, the product is unlikely to qualify as a biosimilar.
- If comparability exercises are not performed as outlined in this document then the final product should not be referred to as a biosimilar.
- Biosimilars are not "generic medicines" and the authorization process for such medicines generally does not apply.
- As with other biological products, biosimilars require effective regulatory oversight pre- and post-approval in order to manage the potential risks they pose and to maximize their benefits.

6. Reference products

Comprehensive information on the reference product (RP) provides the basis for establishing the quality, safety and efficacy profile against which the biosimilar will be compared. The RP also provides the basis for dose selection and route of administration, and is used in the similarity studies required to support the licensing application. Demonstration of a high level of analytical and functional similarity between the biosimilar and RP provides the rationale for a tailored nonclinical and clinical dataset to support the application for market authorization of the biosimilar.

The choice of RP is therefore critically important in the evaluation of a biosimilar. For licensing purposes for a specific biosimilar, a single biological product from one marketing authorization holder should be chosen and defined as the RP.

Traditionally, NRAs have required the use of a nationally licensed RP for the licensing of a generic medicine. In the case of biosimilars, this practice may not always be feasible or necessary, and several regulatory jurisdictions have allowed for the use of a non-local RP as comparator to enable faster development of and access to biological therapies. The use of an RP sourced from another jurisdiction with similar scientific and regulatory standards is therefore possible. The information needed to support the acceptability of an RP sourced from another jurisdiction will be determined by the NRA.

The posology and route of administration of the biosimilar should be the same as that of the RP. However, depending on the jurisdiction, the strength, pharmaceutical form, formulation, excipients and presentation (for example, use of a different medical device or number of syringes in a pack) of the biosimilar might differ from the RP, if justified. The acceptability of additional routes of administration following approval of the biosimilar will also depend upon the jurisdiction.

Since the choice of RP is crucial in the development of a biosimilar, the following should be considered:

- The RP should have been licensed on the basis of a full standalone set of quality, nonclinical, safety and efficacy data (*8*). A biosimilar should therefore not be accepted as an RP.
- There should be sufficient information available to support the safe and efficacious use of the RP.
- For the licensing of a specific biosimilar, a single biological product from one marketing authorization holder should be chosen and defined as the RP. The entire comparability exercise should be performed against this RP. However, as outlined below, if allowed

by the NRA it may be possible to use the same RP sourced from another jurisdiction in clinical studies.

- Where an RP marketed in another jurisdiction (non-local) is allowed by the NRA, the following should be considered:
  - the RP should be licensed in a jurisdiction that has a well-established regulatory framework, as well as experience with the evaluation of biological products and post-marketing surveillance activities; and
  - if the use of a non-local RP containing the same drug substance in clinical studies requires bridging between the local and non-local RPs, suitable analytical and functional bridging data should be provided to demonstrate the representativeness of the non-local RP for the local RP – stringent similarity assessment should be applied for the analytical and functional bridging studies (following the principles provided in sections 7.3 and 7.4 below); additional PK bridging studies may be required, for example if the two RPs have different formulations that may affect PK.
- It is important to note that the acceptance of a non-local RP for the evaluation of a biosimilar in a particular country does not imply that the NRA of that country has approved the RP for use in the domestic market.

7. Quality

The comparison showing molecular similarity between the biosimilar and the RP provides the essential rationale for predicting that the clinical safety and efficacy profiles of the RP apply to the biosimilar. Therefore, a high degree of analytical and functional similarity between the biosimilar and the RP is the basis for developing a biosimilar.

Development of a biosimilar involves the thorough characterization of multiple RP batches in order to obtain an understanding of the overall quality profile as well as the range of variability of the RP batches on the market. Based on the knowledge gained from the RP characterization studies, as well as available in-house and public information, the manufacturing process of the biosimilar is developed to produce a product that is highly similar to the RP in all clinically relevant quality attributes (that is, attributes that may impact clinical performance).

The biosimilar documentation should comply with the standards required by NRAs for originator products. A full quality dossier for both drug substance

and drug product is always required – see relevant guidelines for each class of product, such as those issued by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) and the WHO Guidelines on the quality, safety and efficacy of biotherapeutic protein products prepared by recombinant DNA technology (*8*) and the WHO Guidelines on evaluation of monoclonal antibodies as similar biotherapeutic products (SBPs) (*10*). The manufacturer of the biosimilar should additionally carry out a comprehensive and comparative state-of-the-art physicochemical and biological characterization of the biosimilar and the RP and document the results in the submitted dossier.

7.1 International reference standards

WHO provides international standards (IS) and reference reagents, which serve as reference sources of defined biological activity expressed in International Units (IU) or Units (U). These materials are intended for use in the calibration of bioassays and are available for a wide range of substances including hormones (for example, erythropoietin, follicle-stimulating hormone) and cytokines – for example, granulocyte-colony stimulating factor (G CSF) – as well as modified/long-acting proteins (such as pegylated G-CSF, darbepoetin and etanercept) and monoclonal antibodies (mAbs). IS for the latter product class are expanding and currently include standards for adalimumab, bevacizumab, infliximab, rituximab and trastuzumab.[18] These standards are produced according to defined criteria as per WHO recommendations (*11*) and often contain excipients which optimize the retention of biological activity and other important characteristics as well as ensuring stability, but which may also interfere with physicochemical methods. The standards are important for assay development, for qualifying and validating assays for their intended use, for monitoring the potency of individual/diverse products, for calibrating bioassays (either directly or to calibrate national or pharmacopoeial standards) and for supporting assay performance throughout the life-cycle of a product. In addition, they can be used for the independent testing of falsified medicines and as an independent standard for tracing bioactivity horizontally (between products and batches) and longitudinally (over time) to support post-marketing surveillance activities and to assess any divergence that may occur as the product(s) evolve.

For biological medicines, expression of potency in units of bioactivity relative to an independent standard is an essential regulatory tool for harmonizing product dosing for patients globally. For many years, WHO IS have provided

[18] For the full range of available WHO international biological reference materials please see: https://www.who.int/activities/providing-international-biological-reference-preparations

a mechanism for assigning and maintaining biological potency across diverse products. It should be noted however that with the development of innovative products the role of the IS in potency determination is changing and decisions on potency and labelling are likely to be made on a case-by-case basis depending on the product and the situation that exists when the biosimilar is developed. For example, for naturally derived proteins such as coagulation factors and hormones (for example, erythropoietin and follicle-stimulating hormone), where the establishment of the IS with an assigned IU preceded the development of versions derived from recombinant DNA (rDNA), the practice of using the IU for potency assignment, dosage and product labelling is well established, and where applicable this has continued for biosimilars. However, the situation is different for non-natural and engineered proteins such as mAbs. Since IS did not exist when the innovator products were developed, such products were licensed and marketed for clinical use with potency described by manufacturers in proprietary units relative to their in-house product-specific reference material, with product dosing and labelling given in mass units. The practice of determining potency relative to an in-house qualified reference material and of using mass units for dosing/labelling has also been implemented by biosimilar manufacturers and is expected to continue. In this situation, manufacturers should develop a well-characterized product-specific in-house reference material calibrated against the IS (where this exists) with a regulatory expectation that the implementation and management of this in-house reference material (two-tiered approach) will be conducted as per regulatory guidance. Consistent with the biosimilarity paradigm, the retrospective establishment of an IU value should not affect the potency of the biosimilar (which should be aligned with the RP) and should not affect the labelling or dosing regimens of existing or future products.

It is important to note that WHO IS and other WHO reference standards are not medicinal products (even though the drug substance in them may be derived from material that was produced at clinical grade) and are distinct (for example, in terms of protein content, formulation etc.) from the RP which has an established clinical history and is an essential component of the biosimilarity route to licensure. The RP defines the quality target product profile that a biosimilar must meet as per the principles of biosimilarity – a function that the reference standard does not serve. Instead, the IS defines the IU of bioactivity for the calibration of bioassays (either directly or through the calibration of manufacturer reference materials) and thus plays an essential role in the development of suitable assay methods. It should further be noted that the IS cannot be used to determine a product's specific activity, dictate the quality of acceptable biosimilars for regulatory purposes or demonstrate biosimilarity, and should therefore not be misused as a comparator for biosimilar development (*12–14*). Importantly, the IS: (a) allows for an understanding of consistency in bioactivity across batches

of a product throughout its life-cycle; (b) provides continuity with respect to the in-house reference material and supports transition (change) as the product evolves; (c) facilitates the harmonization of bioactivity across different products (both RPs and biosimilars); and (d) increases confidence in the quality of globally available biosimilars.

7.2 Manufacturing process

The manufacturing process of the biosimilar should be developed based on a comprehensive understanding of the RP gained through detailed characterization studies of a sufficient number of RP batches.

It is understood that a manufacturer developing a biosimilar will not normally have access to confidential details of the RP manufacturing process – thus, the process will differ from the licensed process for the RP. In order to produce a high-quality product as similar as possible to the RP, the biosimilar manufacturer should assemble all available knowledge on the RP regarding the type of host cell, product formulation and the container closure system used for marketing. Although the biosimilar does not need to be expressed in the same type of host cell as that used for the RP, it is recommended that a similar host cell type is used (for example, Escherichia coli, Chinese hamster ovary cells, etc.). This will reduce the potential for critical changes in the quality attributes of the protein, or in post-translational modifications, product-related impurities or the process-related impurity profile, that could potentially affect clinical outcomes and immunogenicity. If a different host cell is used (for example to avoid unwanted and potentially immunogenic glycan structures present in the RP) then changes introduced in terms of product-related substances, as well as product- and process-related impurities, need to be carefully considered.

The manufacturing process used can significantly affect the structure of the drug substance and thereby impact upon the potency of the product. For example, in the case of mAbs, when deciding upon the expression system to employ, manufacturers should be guided by the potential for both enzymatic and non-enzymatic modifications, such as incomplete disulfide bond formation, formation of aggregates, glycosylation, N-terminal pyroglutamine cyclization, C-terminal lysine processing, deamidation, isomerization and oxidation, modification of the N-terminal amino acids by maleuric acid, and amidation of the C-terminal amino acid.

The manufacturer must demonstrate the consistency and robustness of the manufacturing process by implementing state-of-the-art quality control and assurance procedures, in-process controls and process validation. The biosimilar manufacturing process should meet the same standards required for originator products, including manufacture under current good manufacturing practices (*15*, *16*).

As for any biological product, if process changes are introduced during the development of a biosimilar then the impact of the changes should be assessed through a comparability exercise (*9*, *17*). Although many of the same principles are followed, the assessment of manufacturing process changes should be addressed separately from the comparability exercise performed to demonstrate biosimilarity with the RP (see section 7.4 below). It is, however, strongly recommended that the pivotal data used to demonstrate biosimilarity are generated using biosimilar batches manufactured using the commercial manufacturing process and therefore representing the quality profile of the batches to be commercialized.

7.3 Analytical considerations

Thorough characterization of both the RP and the biosimilar should be carried out using state-of-the-art chemical, biochemical, biophysical and biological analytical techniques. The methods should be scientifically sound and demonstrated to be of appropriate sensitivity and specificity for their intended use.

Details should be provided on primary and higher-order structure, post-translational modifications (including, but not limited to, glycoforms), biological activity, purity, impurities, product-related (active) substances (variants) and immunochemical properties, where relevant. Orthogonal methods should be used, as far as possible – that is, the variants and quality attributes of the product should be analysed using analytical methods with different underlying chemical, physical and biological properties. For example, ion exchange chromatography, isoelectric focusing and capillary electrophoresis all separate proteins based upon charge but do so under different analytical conditions and on the basis of different physicochemical properties of the biological product. As a result, one method may detect variants that another method does not. The goal of the comparability investigation is to be as comprehensive as possible in order to minimize the possibility of undetected differences between the RP and the biosimilar that may affect safety and clinical activity. The analytical limitations of each technique (for example, limit of detection or resolving power) should be considered when determining the similarity of a biosimilar to its RP.

Representative raw data should be provided for analytical methods (for example, high-quality reproductions of gels and chromatograms) in addition to tabular data summarizing the complete dataset and showing the results of all release and characterization analyses carried out on the biosimilar and the RP. Graphical presentation of datasets comparing biosimilar and RP analytical data should also be produced where possible. The results should be accompanied by sufficient interpretation and discussion of the findings.

The measurement of quality attributes in characterization studies (as opposed to batch release tests) does not necessarily require the use of validated

assays, but the assays used should be scientifically sound and qualified – that is, they should provide results that are meaningful and reliable. The methods used to measure quality attributes for batch release should be validated in accordance with relevant guidelines, as appropriate. A complete description of the analytical techniques employed for release and characterization of the product, along with method validation or qualification data (as appropriate), should be provided in the licence application.

Due to the unavailability of drug substance for the RP, the biosimilar manufacturer will usually be using a commercial drug product for the similarity exercise. The commercial drug product will, by definition, be in the final dosage form containing the drug substance(s) formulated with excipients. It should be verified that these excipients do not interfere with the analytical methods used and thus have no impact on test results. If the drug substance in the RP needs to be purified from a formulated reference drug product in order to be suitable for characterization then studies must be carried out to demonstrate that product heterogeneity and relevant attributes of the active moiety are not affected by the isolation process. The approach used for isolating the drug substance of the RP and comparing it with the biosimilar should be justified and demonstrated (with accompanying data) to be appropriate for the intended purpose.

7.3.1 Physicochemical properties

The physicochemical characterization should include determination of primary and higher-order structure (secondary/tertiary/quaternary) and product variants using appropriate analytical methods (for example, mass spectrometry, circular dichroism, spectroscopy etc.) as well as other biophysical properties.

The amino acid sequence of a biosimilar should be confirmed to be the same as that of its RP. It is, however, further recommended that manufacturers pay special attention to any sequence variants present in the biosimilar. Although an identical primary sequence between the biosimilar and the RP is expected, low-level sequence variants may occur due to transcription and translation errors, especially through amino acid misincorporation during high-level expression, and should be identified if present. The presence of such variants could be acceptable if properly described and controlled to a reasonable level. An assessment of the potential clinical impact of such variants would also need to be considered.

An inherent degree of structural heterogeneity occurs in proteins as a result of biosynthesis processes. These include C-terminal processing, N-terminal pyroglutamation, deamidation, oxidation, isomerization, fragmentation, disulfide bond mismatch and free sulfhydryl groups, N-linked and O-linked oligosaccharide, glycation and aggregation. The structural heterogeneity present in the biosimilar should be evaluated relative to the RP. Experimentally

determined disulfide bonding patterns should be compared to the predicted structure based on well-established structural data on the molecule.

7.3.2 Biological activity

Biological activity is the specific ability or capacity of the product to achieve a defined biological effect. It serves multiple purposes in the assessment of product quality and is required for characterization (see also section 8 below) and for batch analysis. Ideally, the biological assay used will reflect the understood mechanism of action of the drug substance of the RP and will thus serve as a link to clinical activity. A biological assay is a quality measure of the activity of the drug substance and can be used to determine whether a product variant is active (that is, a product-related substance) or inactive (and therefore defined as an impurity). Biological assays can also be used to confirm that small differences observed in the higher-order structure of a molecule have no influence on its biological activity. Thus, the use of relevant biological assay(s) of appropriate precision, accuracy and sensitivity provides an important means of confirming that there is no significant functional difference between the biosimilar and the RP.

For a product with multiple biological activities, manufacturers should perform, as part of product characterization, a set of relevant functional assays designed to evaluate the range of activities of the product. For example, certain proteins possess multiple functional domains that express enzymatic and receptor-binding activities. In such situations, manufacturers should evaluate and compare all relevant functional activities of the biosimilar and the RP.

Potency is the measure of the biological activity. The potency assay should be used together with an in-house qualified reference material that is representative of the biosimilar material. The use of the IS for determining potency depends on the prevailing practice for the product. Where appropriate, international or national standards and reference reagents should be used to determine product potency and to express results in IU – for other products, a suitable in-house reference material should be used (see section 7.1 above). In-house reference materials should be quantitatively calibrated against either an international or national standard or reference reagent, where available and appropriate.

Depending on the purpose of the method (batch release assay or characterization), the functional assays used may or may not be fully validated, but they must be scientifically sound and produce consistent and reliable results. The available information on these assays (including extent of validation, assessed parameters and available validation data) should be confirmed before they are applied to the testing and establishing of biosimilarity between a biosimilar and its RP. It should be noted that many biological assays may have relatively high variability that might preclude detection of small but significant differences between the biosimilar and RP. Therefore, it is recommended that

assays are developed that are more precise and can detect changes in the intended biological activities of the product to be evaluated with adequate accuracy. Such assays can include target-binding assays (which are usually less variable) in addition to cell-based assays. Adopting automated laboratory equipment to help minimize manual operations, applying good analytical practices and appropriate control sampling, and using critical reagents calibrated against WHO or national reference standards where available (for example, tumour necrosis factor alpha (TNF-α) for potency assays for anti-TNF products) may help to reduce the variability of biological assays. For a given method variability, the number of RP batches tested should be high enough to allow for a reliable assessment of similarity (see section 7.4.1 below).

When immunochemical properties are part of the activity attributed to the product (for example, antibodies or antibody-based products) analytical tests should be performed to characterize these properties and used in the comparative studies. For mAbs, the specificity, affinity and binding kinetics of the product to relevant fragment crystallizable (Fc) receptors (for example, neonatal Fc receptor, complement component 1q (C1q) and Fcγ receptors) should be compared using suitable methods such as surface plasmon resonance and biolayer interferometry. In addition, appropriate assays should be used to provide information on Fc-mediated functions – for example, antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP) and complement-dependent cytotoxicity (CDC), where relevant.

The correlation between Fc-mediated effector functions, Fcγ receptor or C1q binding and physicochemical characteristics (for example, glycan pattern) should be considered and, whenever possible, established. Such analyses will facilitate the interpretation of subtle differences between the biosimilar and the RP and inform prediction of their clinical impact.

7.3.3 Purity and impurities

Product- and process-related impurities should be identified and quantified using orthogonal and state-of-the-art technologies.

Product-related substances and impurities, such as those caused by protein degradation, oxidation, deamidation, aggregation or potential post-translational modification of the protein, should be compared for the biosimilar and RP. If comparison reveals differences in product-related substances and impurities between the biosimilar and RP, the impact of the differences on the clinical performance of the drug product (including its biological activity) should be evaluated. Specifically, if the manufacturing process used to produce the proposed biosimilar introduces different impurities or higher levels of impurities than those present in the RP then additional functional assays to evaluate the impact of the differences may be necessary (see section 7.4.2 below). To obtain

sufficient information of the product-related substances and impurities it is recommended that comparative stability studies under accelerated and/or stress conditions are conducted (see section 7.6 below).

Process-related impurities such as host cell proteins, host cell DNA, cell culture residues and downstream processing residues may be quantitatively and/or qualitatively different between the biosimilar and RP due to the different manufacturing processes used for their drug products. Nevertheless, process-related impurities should be kept to a minimum through the use of state-of the-art manufacturing technologies. The risk related to any newly identified impurities in the biosimilar should be evaluated.

7.3.4 Quantity

In general, a biosimilar is expected to have the same concentration or strength of the drug substance as the RP. Depending on the jurisdiction, concentration deviations not affecting the posology might be permissible, if justified (see section 8 below). The quantity of the biosimilar drug substance should be expressed using the same measurement system as that used for the RP (that is, mass units or units of activity). A description with appropriate justification should also be included to describe how the quantity was calculated (including, for example, the selection of the extinction coefficient).

7.4 Comparative analytical assessment

7.4.1 Considerations for the RP and the biosimilar

The number of RP batches needed for the comparative analytical assessment will be influenced by the criticality of the quality attribute(s) under investigation and the approach chosen for demonstrating similarity. The manufacturer of the biosimilar should include an appropriate and scientifically supportable number of batches of the RP in the comparability assessment. In order to characterize independent RP batches, it is recommended that the RP batches are sourced over an extended time period. These batches should also include the RP batches used in the clinical comparison studies of the biosimilar. In general, sampling a higher number of RP batches will provide a better estimate of the true batch-to-batch variability of the RP and allow for a more robust statistical comparison with the biosimilar.

Random sampling of RP batches is desirable but may be difficult to achieve in practice depending on the availability of such batches. However, the sourcing of RP batches should be carefully managed to generate a sample that captures the inherent variability of the RP (for example, collected over a sufficient timeframe with the aim of covering different manufacturing campaigns). The RP batches should be transported and stored under the recommended conditions

and tested within their approved shelf-life. Any exception to this would have to be fully substantiated with experimental data. The shelf-life of the RP at time of characterization should be considered and it is expected that RP batches of different ages will be included in the similarity assessment.

The biosimilar batches included in the comparability assessment should be manufactured using the intended commercial manufacturing process and should preferably originate from different drug substance batches. Generally, each value for an attribute being assessed for a biosimilar should be contributed by an independent batch. For example, a single drug product batch produced from a single drug substance batch would be considered to be an independent batch while different drug product batches produced from the same drug substance batch cannot be considered to be independent. In addition, small- or pilot-scale batches can be included if comparability between the small- and commercial-scale batches has been properly demonstrated. Usually all commercial-scale batches produced – including process performance qualification batches and batches applied in the clinical trial(s) – should be included in the similarity assessment. As with the RP, the exact number of biosimilar batches required will be influenced by several factors, such as the criticality of the quality attribute(s) under investigation and the approach applied for similarity evaluation. In general, the risk of a false-positive conclusion on similarity will decrease with increasing number of batches. A robust manufacturing control system and demonstrated batch-to-batch consistency of the biosimilar (see section 7.2 above) are prerequisites for a successful similarity assessment.

7.4.2 Considerations for similarity assessment

Prior to initiating the comparability exercise, it is recommended that the quality attributes of the RP are identified and ranked according to their impact on the clinical performance of the product. For this purpose, a risk ranking tool could be developed. Such risk ranking tools should consider the impact of the quality attribute on safety, efficacy, PK and immunogenicity. Furthermore, the degree of uncertainty of impact should be taken into consideration. If it is known that a quality attribute will impact the clinical performance (that is, the uncertainty is low but the impact high) then that quality attribute should be prioritized and the overall risk score should be high. In cases where the clinical relevance of a certain quality attribute is unknown (that is, the uncertainty is high) then higher risk scores should be assigned even to lower impact quality attributes. Further guidance on the use of risk ranking tools can be found in national and international guidelines (*18*).

The result of the risk ranking could then be used to guide the data analyses and the overall assessment of similarity. The most frequently used approach for similarity assessment relies on demonstrating that the quality attributes of the

biosimilar batches lie within the predetermined similarity ranges established based on characterization data from multiple batches of the RP. Other approaches (such as equivalence testing of means) can also be used for similarity assessment. Each statistical approach has, however, specific strengths and weaknesses which should be appropriately discussed in the submission and considered in the similarity conclusion. In order to mitigate the risks inherent in employing statistical tests on limited samples (false-positive and false-negative conclusions), a comprehensive control strategy must be established for the biosimilar to ensure consistent manufacturing.

7.4.2.1 Statistical intervals for the establishment of similarity ranges

Where possible, quantitative similarity ranges should be established for the biosimilar comparability exercise. As the allowable differences in quality attributes between the biosimilar and the RP are usually difficult to establish based on clinical considerations alone, the batch-to-batch variability of the RP is typically used to further inform acceptable differences in quality attributes. The established similarity range should therefore tightly reflect the quality profile of the marketed RP batches. The ranges should normally not be wider than the batch-to-batch variability present in the RP unless it can be determined which differences would be acceptable (for example, less impurities is usually acceptable). Wide similarity ranges based on inappropriate use of statistical methods should not be used.

Different statistical intervals can be used to establish similarity ranges. Commonly used approaches include mean ± x SD, the min-max range and tolerance intervals:

- The most commonly applied approach for establishing similarity ranges is the x-sigma interval, that is, mean ± x SD of the RP batch data. The multiplier used (x) should be scientifically justified and could be linked to the criticality of the quality attribute tested, with a smaller multiplier applied for high criticality quality attributes.
- A conservative approach would be to establish the similarity ranges directly based on the min-max quality attribute data obtained from the characterization studies of RP batches. Such similarity ranges could be viewed as clinically qualified (since the RP batches are on the market and taken by patients). However, compared to other approaches the min-max approach is often associated with high risk of a false-negative conclusion (that is, a high risk of concluding non-similarity even though the underlying data distributions for the RP and biosimilar would support a similarity claim).
- Similarity ranges based on tolerance intervals would usually require a high number of RP batches for establishing meaningful

ranges. With a limited number of RP batches characterized and/or inappropriate parameterization, the tolerance interval approach can result in an estimated range that is much wider than the actual min-max quality attribute ranges of the RP. The risk of a false-positive conclusion of similarity (that is, the risk of concluding similarity where the underlying data distributions do not support such a claim) may therefore be unreasonably high when the similarity ranges are based on inappropriately applied tolerance intervals.

The most frequently applied overall similarity criteria require that a certain percentage of the biosimilar batches (usually between 90% and 100%) fall within the similarity range. This figure should be determined prior to the initiation of the similarity assessment.

7.4.2.2 Analytical similarity evaluation

It is up to the manufacturer to justify the relevance of the established similarity ranges and criteria. Ideally, the data analyses should be robust and should as far as possible minimize the risk of a false-positive conclusion. In some jurisdictions, the use of a stringent similarity evaluation could also allow for discussion with the NRA on further tailoring of the clinical comparability programme. Although decreasing the risk of a false-positive conclusion is of primary importance from a patient and regulatory point of view, the risk of a false-negative conclusion also needs to be managed by the manufacturer and should be thoroughly considered during the planning of the similarity exercise.

Some minor differences between the RP and the biosimilar are expected. Nevertheless, any quality attributes not fulfilling the established similarity criteria should be considered as a potential signal for non-similarity and should be assessed for possible impact on clinical safety and efficacy. Confirmed differences in low criticality quality attributes also need to be adequately considered, but in the case of such differences reference to available information (which could, for example, originate from scientific publications) is usually sufficient. Lower impurity levels in the biosimilar (for example, of aggregates) or differences in quality attributes present at very low levels in both the RP and the biosimilar would in most cases be predicted to have no clinical relevance, and could therefore be accepted without further assessment. For differences in quality attributes with higher criticality, functional assays to thoroughly address their possible clinical impact are generally expected. Where there are confirmed differences in the most critical quality attributes it will be more challenging to justify the conclusion that the product is a true biosimilar. For example, if differences are found in quality attributes that alter the PK of the product and thereby change the dosing scheme then the product cannot be considered to be a biosimilar.

7.5 Specifications

Specifications are employed to verify the routine quality of the drug substance and drug product rather than to fully characterize them. As for any biological product, the specifications for a biosimilar should be set as described in established guidelines. Furthermore, a biosimilar should show the same level of compliance with a pharmacopoeial monograph as that required for the RP – however, compliance with a pharmacopoeial monograph is not sufficient to establish biosimilarity. It should also be noted that pharmacopoeial monographs may provide only a minimum set of requirements for a particular product, and specification of additional test parameters may be required. Reference to the analytical methods used and acceptance limits for each test parameter of the biosimilar should be provided and justified. All analytical methods referenced in the specification should be validated and the corresponding validation documented.

Specifications for a biosimilar may not be the same as for the RP since the manufacturing processes will be different, and different analytical procedures and laboratories will be used for the assays. Nonetheless, the specifications should capture and control important known product quality attributes. The setting of specifications should be based on: (a) the manufacturer's experience with the biosimilar (for example, with regard to its manufacturing history, assay capability and the quality profile of batches used for establishing similarity); (b) the experimental results obtained by testing and comparing the biosimilar and RP; and (c) attributes with potential impact on product performance. The manufacturer should take into consideration that the limits set for a given specification should not, unless properly justified, be significantly wider than the range of variability of the RP over the shelf-life of the product.

7.6 Stability

Stability studies should comply with relevant guidance as recommended by the NRA. Generally, stability studies should be summarized in an appropriate format (such as tables) and should include results from accelerated degradation studies and studies under various stress conditions (for example, high temperature, oxidation, freeze-thaw, light exposure, humidity and mechanical agitation). There are a number of specific reasons for performing stability studies:

- First, the stability data should support the conclusions reached on the recommended storage and shipping conditions, and on the shelf-life and storage period for the drug substance, drug product and process intermediates – which might be stored for significant periods of time. Real-time/real-temperature stability studies will determine the storage conditions and shelf-life for the biosimilar –

which may or may not be the same as those for the RP. Results from studies conducted under accelerated and stress conditions may also show that additional controls should be used in the manufacturing process, and during shipping and storage, in order to ensure the integrity of the product.

- Secondly, stability studies should be carried out to show which release and characterization methods are stability-indicating for the product.
- Thirdly, comparative stability studies conducted under accelerated, and in some cases stress conditions (for example, freeze-thaw, light exposure and mechanical agitation), can be valuable in determining the similarity of the products by showing a comparable degradation profile and rate, with formulation, volume, concentration and/or container differences taken into account.

Stability studies on the drug substance should be carried out using containers and conditions that are representative of the actual storage containers and conditions. Stability studies on the drug product should be carried out in the intended drug product container closure system.

8. Nonclinical evaluation

This section addresses the pharmaco-toxicological assessment of the biosimilar. It is important to note that in order to design an appropriate nonclinical study programme a clear understanding of the characteristics of the RP is required.

The nature and complexity of the RP will have an impact on the extent of the nonclinical studies needed to confirm biosimilarity. In addition, any differences observed between the biosimilar and RP in the physicochemical and biological analyses will also guide the planning of the nonclinical studies. Other factors that need to be taken into consideration include the mechanism(s) of action of the drug substance (for example, the receptor(s) involved) in all authorized indications of the RP, and the pathogenic mechanisms involved in the disorders included in the therapeutic indications.

A stepwise approach should be applied during nonclinical development to evaluate the similarity of the biosimilar and its selected RP. At first, in vitro studies should be conducted and then a decision made on whether or not additional in vivo animal studies are required.

The following approach to nonclinical evaluation may be considered and should be tailored on a case-by-case basis to the biosimilar concerned. In all cases, the approach chosen should be scientifically justified in the application dossier.

8.1 In vitro studies

In order to assess any relevant difference in pharmaco-toxicological activity between the biosimilar and chosen RP, data from a number of comparative in vitro studies – some of which may already be available from the quality-related assays – should be provided. In light of this data overlap, it is suggested that the in vitro nonclinical studies related to characterization of the biological activity of the biosimilar be addressed alongside the related quality data in the corresponding quality module (see section 7.3.2 above). Any other nonclinical in vitro studies should then be addressed in the relevant nonclinical modules of the dossier where they should be reviewed and discussed from the point of view of potential impact on the efficacy and safety of the biosimilar.

Since experience has shown that in vitro assays are in general more specific and sensitive than in vivo studies in animals for detecting differences between the biosimilar and RP, the use of in vitro assays is of paramount importance in the nonclinical biosimilar comparability exercise.

For such in vitro studies, the following general principles apply:

- Typically, a battery of interaction studies addressing the primary binding events should be performed, along with cell-based or isolated-tissue-based functional assays (see below) in order to assess if any (clinically) relevant differences in reactivity exist between the biosimilar and RP and, if so, to determine the likely causative factor(s).
- Together, these assays should cover the whole spectrum of pharmaco-toxicological aspects with potential clinical relevance for the RP and for the product class. In the dossier, the manufacturer should discuss to what degree the in vitro assays used can be considered representative/predictive of the clinical situation according to current scientific knowledge.
- The studies should be comparative and designed to be sufficiently sensitive, specific and discriminatory to allow for the detection of (clinically) relevant differences in pharmaco-toxicological activity between the biosimilar and RP – or, conversely, to provide evidence that any observed differences in quality attributes are not clinically relevant.
- The studies should compare the concentration–activity/binding relationship of the biosimilar and the RP at the pharmacological target(s), covering a concentration range within which potential differences are most accurately detectable (that is, the ascending part of the concentration–activity/binding curve).
- A sufficient number of RP batches and biosimilar batches (preferably representative of the material intended for commercial use) should

be evaluated. Assay and batch-to-batch variability will affect the number of batches needed. The number tested should be sufficient to draw meaningful conclusions on the variability of a given parameter for both the biosimilar and the RP and on the similarity of both products (see section 7.4.1 above).

- Where available, international reference standards can be used to support assay characterization, calibration and performance (see section 7.1 above). When no such reference standard exists, an in-house reference material should be established.

The nonclinical in vitro programme for biosimilars should usually include relevant assays for the following:

- **Binding studies**
  Evaluation of the primary binding events – that is, binding of the biosimilar to cell membrane receptors or to other membrane-bound or soluble targets that are known/assumed to be involved in the pharmaco-toxicological effects of the RP in the clinically approved indications – for example, for immunoglobulin G (IgG)-based mAbs, antigen-binding fragment (Fab)-associated binding to the antigen and Fc-associated binding to representative isoforms of the relevant Fc receptors and to C1q – see (*10*).
- **Functional studies/determination of biological activities**
  Studies should evaluate signal transduction and/or functional activity/viability of cells or isolated tissues known to be of relevance for the pharmaco-toxicological effects of the RP. Together these assays should broadly cover all the known mechanisms of action of the RP in the clinically authorized indications – for example, for IgG-based mAbs directed against membrane-bound antigens, evaluation of Fab-associated functions and of Fc-associated functions such as ADCC, ADCP and CDC – see (*10*).

Such assays are often technically demanding and the experimental approach chosen should be appropriately justified by the manufacturer.

For additional guidance on these topics see section 7.3 above.

8.2 Determination of the need for in vivo animal studies

On the basis of the totality of quality and nonclinical in vitro data available and the extent to which there is residual uncertainty about the similarity of a biosimilar and its RP, it is at the discretion of the involved NRA to waive or not to waive a requirement for additional nonclinical in vivo animal studies. The decision of

the NRA on whether or not to require such studies should take into account the following:

- If the quality comparability exercise and the nonclinical in vitro studies have shown high similarity and the level of residual uncertainty is considered acceptable to move to the clinical phase of the similarity exercise then an additional in vivo animal study is not considered necessary.
- If a need is identified to reduce remaining uncertainties concerning the similarity (including drug safety) of a biosimilar and its RP before the initiation of clinical evaluations then additional in vivo animal studies may be considered, if a relevant animal model is available – however this should only occur: (a) when it is expected that such studies would provide relevant additional information; and (b) if the needed additional information cannot be obtained using an alternative approach that does not involve in vivo animal studies. In this respect, the factors to be considered could include:
  - qualitative and/or quantitative differences in potentially or known relevant quality attributes between the biosimilar and its RP (for example, qualitative and/or quantitative differences in the post-translational glycosylation of proteins); and
  - relevant differences in formulation (for example, use of excipients in the biosimilar not widely used in medicinal products).
- On the basis of regulatory experience gained to date in marketing authorization applications for biosimilars, the need for additional in vivo animal studies would be expected to represent a rare scenario.
- If the quality and nonclinical in vitro comparability exercises indicate relevant differences between the biosimilar and the RP (thus making it unlikely that biosimilarity would eventually be established), then standalone development to support a full marketing authorization application should be considered instead (see section 5 above).

8.3 In vivo studies

8.3.1 General aspects to be considered

In the exceptional case that an in vivo evaluation is deemed necessary by the involved NRA, the focus of the study/studies (PK and/or PD and/or safety) will depend upon the type of additional information needed.

Animal studies should be designed to maximize the information obtained. The 3Rs principles for animal experiments (Replace, Reduce, Refine) should always be followed to minimize the use of animals in testing.

To address the residual uncertainties, the use of conventional animal species and/or of specific animal models (for example, transgenic animals or transplant models) may be considered.

Animal models are often not sensitive enough to detect small differences. If a relevant and sufficiently sensitive in vivo animal model cannot be identified, the manufacturer may choose to proceed directly to clinical studies, taking into account strict principles to mitigate any potential risk.

The effects of RPs are often species specific. In accordance with ICH S6(R1) (*19*) and the WHO Guidelines on the quality, safety and efficacy of biotherapeutic protein products prepared by recombinant DNA technology (*8*), in vivo studies should be performed only in relevant species – that is, species which are known to be pharmacologically and/or toxicologically responsive to the RP.

The duration of the study/studies should be justified, taking into consideration the PK behaviour of the RP, the time to onset of formation of anti-drug antibodies (ADAs) in the test species and the clinical use of the RP.

8.3.2 Specific aspects

8.3.2.1 PK and/or PD studies

In cases where such studies are considered necessary, the PK and/or PD of the biosimilar and the RP should be compared quantitatively, when the model allows, using a dose–response assessment that includes the intended exposure in humans.

The studies may include animal models of disease to evaluate functional effects on disease-related PD markers or efficacy measures.

8.3.2.2 Safety studies

Where in vivo safety studies are deemed necessary, a flexible approach that follows the 3R principles to maximize the readout of relevant data and minimize the use of animals in testing should always be followed. If appropriately justified, a repeated dose toxicity study with refined design – for example, using just one dose level of biosimilar and RP, and/or just one gender and/or no recovery animals, and/or only in-life safety evaluations such as clinical signs, body weight and vital functions – may be considered. Depending on the chosen end-points, it may not be necessary to sacrifice the animals at the end of the study.

Repeated dose toxicity studies in non-human primates are not recommended and nor are toxicity studies in non-relevant species (for example, to assess unspecific toxicity due to impurities).

8.3.2.3 Immunogenicity studies

Qualitative or quantitative difference(s) in product-related variants (for example, in glycosylation patterns, charge, aggregates, and impurities such as host-cell proteins) may have an effect on immunogenic potential and on the potential to cause hypersensitivity. These effects are usually difficult to predict from animal studies and are better assessed in clinical studies.

However, determination of antibody formation against the study drugs may be required for the interpretation of PK/toxicokinetic data in cases where in vivo animal studies are needed.

8.3.2.4 Local tolerance studies

Studies on local tolerance are usually not required. However, if excipients are introduced for which there is little or no experience with the intended clinical route of application, local tolerance may need to be evaluated. If other in vivo animal studies are to be conducted, the evaluation of local tolerance may be integrated into the design of those studies.

8.3.2.5 Other studies

In general, safety pharmacology and reproductive and development toxicity studies – as well as genotoxicity and carcinogenicity studies; see (*8*) and (*19*) – are not warranted during the nonclinical testing of biosimilars.

9. Clinical evaluation

The main clinical data should be generated using the biosimilar product derived from the final manufacturing process, and which reflects the product for which marketing authorization is being sought. Any deviation from this recommendation needs to be justified and additional data may be required. For changes in the manufacturing process, relevant guidelines should be followed (*9*, *17*). Ideally, an RP from a single marketing authorization holder would be used as the comparator throughout the comparability programme of quality and clinical studies during the evaluation of the biosimilar in order to allow for the generation of coherent data and conclusions.

Clinical studies are a valuable step in confirming similarity. The goal of such studies is to confirm the absence of any clinically relevant differences between the proposed biosimilar and the RP.

Clinical studies should be designed to demonstrate confirmative evidence of the similar clinical performance of the biosimilar and the RP, and therefore need to use testing strategies that are sufficiently sensitive to detect any clinically relevant differences between the products.

If relevant differences between the biosimilar and the RP are detected at any stage of development, the reasons will need to be explored and justified. If this is not possible, the new product may not qualify as a biosimilar and a full licensing (standalone) application should be considered.

A comparative bioequivalence study involving PK and/or PD comparability is generally required for clinical evaluation. An adequately powered comparative efficacy and safety trial will not be necessary if sufficient evidence of biosimilarity can be drawn from other parts of the comparability exercise. The need for a comparative clinical efficacy and safety trial for the proposed biosimilar (and type of trial if required) will be influenced by factors such as:

- how well the biosimilar can be characterized;
- the availability of suitable, sensitive and orthogonal assays for adequate analytical and functional characterization;
- the degree of analytical and functional similarity between the biosimilar and RP;
- the existence of a relevant PD parameter;
- the degree of understanding of the mechanism(s) of action of the biological product in different indications and how well these can be investigated in binding and functional in vitro tests – the contribution of each mechanism of action to the observed clinical effect is not relevant as long as it can be measured;
- knowledge of any (potentially) unwanted immunogenicity – for example, ADA incidence and the magnitude of ADA response including level of neutralizing antibodies, and antibodies targeting endogenous substances (for example, erythropoietin and coagulation factors); and
- whether the impurity profile or the nature of excipients of the biosimilar gives rise to clinical concerns.

Current examples of biological products that can be comprehensively characterized and have a well-established mechanism of action include (but are not limited to) teriparatide, insulin, G-CSF and somatropin (*20*, *21*). The current data suggest that more-complex products such as mAbs can be sufficiently characterized by available suitable analytical methods, plus the structure–function relationships are well known and can be studied by sensitive orthogonal functional assays (*22*).

9.1 Pharmacokinetic studies

The clinical comparability exercise should generally include a comparative PK study, if the drug substance can be measured in the blood, and should also include the measurement of PD markers if available and also immunogenicity data.

The PK study should be designed to demonstrate similar PK profiles for the biosimilar and the RP. When the RP and its proposed biosimilar have more than one route of administration (most commonly intravenous and subcutaneous) then carrying out the study/studies using the non-intravenous route of administration is preferred as this is usually the more immunogenic route and will provide more meaningful information for the comparability exercise. The omission of a PK study of other approved routes of administration needs to be justified for approval of all available options – for example, in cases when the molecule has an absorption constant that is much lower than the elimination constant (flip flop kinetics).

The sample size should be appropriate, taking into account PK variability in the study population, and consideration should be given to whether a cross-over or parallel group design would be the most adequate. If appropriate population PK or PK-PD models are available for the RP in the literature, modelling and simulation can be considered for optimizing study design – for example, justification of dose(s) and selection of the most sensitive study population to detect potential PK differences, and choice of sample size.

PK studies should preferably be performed in healthy volunteers (if considered ethical) and care should be taken to standardize the population with regard to factors that may influence variability (for example, ethnic origin, body weight and gender). If the drug substance under investigation is associated with risks or tolerability issues that are considered to be unacceptable for healthy volunteers, it will be necessary to perform the PK studies in patients.

The preferred design is a randomized, two-period, two-sequence, single-dose cross-over PK study using a dose within the therapeutic range at which the ability to detect differences is sufficient to observe meaningful differences. The cross-over design eliminates inter-subject variability and therefore (compared with the parallel group design) reduces the sample size needed to show equivalent PK profiles of the biosimilar and RP. The treatment periods should be separated by a wash out phase that is sufficiently long to ensure that drug concentrations are below the lower limit of bioanalytical quantification in all subjects at the beginning of the second period – that is, at least 5 times the terminal half-life.

When a cross-over design is not suitable (for example, for biological products with a very long half-life or associated with immunogenicity affecting PK) then a parallel group study should be considered. In parallel group studies, care should be taken to avoid any imbalances between treatment groups that might affect the PK of the drug substance under investigation (for example, with regard to ethnic origin, body weight and gender).

A multiple-dose study in patients is acceptable as a pivotal PK study if a single-dose study cannot be conducted in healthy volunteers due to risks or tolerability reasons or if a single-dose study is not feasible in patients. Multiple-

dose studies may also be acceptable in rare situations where problems with the sensitivity of the analytical method preclude sufficiently precise plasma or serum concentration measurements after a single dose administration. However, given that a multiple-dose study is less sensitive in detecting differences in Cmax than a single-dose study, this will only be acceptable with sound justification.

PK comparison of the biosimilar and the RP should not only include the rate and extent of absorption but also a descriptive analysis of elimination characteristics – that is, clearance and/or elimination half-life – which might differ between the biosimilar and the RP. Linear (nonspecific) clearance and nonlinear (target-mediated) clearance should be evaluated by assessment of partial areas under the curve (pAUCs). For further details on primary and secondary endpoints for single- and multiple-dose PK studies, please refer to further guidance documents (*23*).

Acceptance criteria for the demonstration of PK similarity between the biosimilar and the RP must be predefined and appropriately justified. It should be noted that the criteria used in standard clinical PK comparability studies (bioequivalence studies) may not necessarily be applicable to all biotherapeutic products. However, the traditional 80–125% equivalence range will in most cases be sufficiently conservative to establish similar PK profiles (*24*). Correction for protein content may be acceptable on a case-by-case basis if pre-specified and adequately justified, with the assay results for the biosimilar and RP being included in the protocol. If adjustments for covariates are intended for parallel group studies (for example, in the case of adalimumab, stratification for body weight and gender), they should be predefined in the statistical analysis plan rather than being included in post hoc analyses.

Other PK studies, such as interaction studies (with drugs likely to be used concomitantly) or studies in special populations (for example, children, the elderly and patients with renal or hepatic insufficiency), are not required for a biosimilar.

Particular consideration should be given to the analytical method selected and its ability to detect and follow the time course of the protein in a complex biological matrix that contains many other proteins. The method should be optimized to provide satisfactory specificity, sensitivity and a range of quantification of adequate accuracy and precision. The same assay should be used to detect the serum concentrations of both the biosimilar and RP. A single PK assay (same binding reagents and a single analytical standard, usually a biosimilar) for determining biosimilar and RP concentration in a biological matrix can be adopted based on verification of the bioanalytical comparability of the two products within the method, with supporting data (*25*).

In some cases the presence of measurable concentrations of endogenous protein may substantially affect the measurement of the concentration–time

profile of the administered exogenous protein. In such cases the manufacturer should describe and justify the approach taken to minimize the influence of the endogenous protein on the results (for example, baseline correction).

In some cases it may not be possible or meaningful to establish PK similarity due to the nature of the substance (for example, fractionated and unfractionated heparin cannot be measured in blood), the route of administration (for example, intraocular administration of aflibercept or ranibizumab) or unacceptably high PK variability (for example, romiplostim). In such cases clinical similarity should be supported by PD, immunogenicity and/or other clinical parameters.

9.2 Pharmacodynamic studies

PD parameters should preferably be investigated as part of the comparative PK studies. In some cases PK studies cannot reasonably be conducted and PD markers may then play a more important role. This is for example the case with heparins,[19] where serum concentrations cannot be measured and similarity needs to be established for the most important PD end-points; that is, at least anti-FXa and anti-FIIa activity.

PD effects should be investigated in a suitable population using a dose or doses within the steep part of the dose–response curve in order to maximize the chance of detecting potential differences between the biosimilar and the RP. PD markers should be selected on the basis of their clinical relevance.

9.3 Confirmatory PK and/or PD studies

If an adequately powered comparative efficacy trial is not necessary, comparative PK (see section 9.1 above) and/or PD studies (see section 9.2 above) may be sufficient for establishing confirmative evidence of the similar clinical performance of a biosimilar and its RP, provided that (*24*):

- the acceptance ranges for confirmatory PK and/or PD end-points are predefined and appropriately justified;
- the PD biomarker reflects the mechanism of action of the biological product;
- the PD biomarker is sensitive to potential differences between the proposed biosimilar and the RP; and
- the PD biomarker assay is validated.

The applicant should consider the option of using additional PD measures (usually as secondary end-points) to assess the comparability of the PD properties

[19] Regulated as a biological in most countries (*1*).

of the RP and proposed biosimilar. Furthermore, even if relevant PD measures are not available, sensitive PD end-points may be assessed if such assessment may help to reduce residual uncertainty about biosimilarity.

An example of acceptable confirmatory PK/PD studies would be the use of euglycaemic clamp studies to compare the efficacy of two insulins. In addition, absolute neutrophil count and CD34+ cell count are the relevant PD markers for assessing the activity of G-CSF and could be used in PK/PD studies in healthy volunteers to demonstrate the similar efficacy of two medicinal products containing G-CSF.

The study population and dosage should represent a test system that is known to be sensitive in detecting potential differences between a biosimilar and the RP. In the case of insulin, for example, the study population should consist of non-obese healthy volunteers or patients with type 1 diabetes rather than insulin-resistant obese patients with type 2 diabetes. Otherwise, it may be necessary to investigate more than one dose to demonstrate that the test system is discriminatory (*26*).

The acceptance ranges for confirmatory PK and/or PD parameters (that is, for primary end-points) should be predefined and appropriately justified. If PD comparison is not essential for a conclusion of biosimilarity but the results are still expected to reasonably support biosimilarity then a purely descriptive analysis of the PD results may be justified. This may be the case for biological substances that have been extensively characterized and for which biosimilarity can already be concluded from the analytical, functional and PK comparisons. If appropriately designed and performed, such PK/PD studies are usually more sensitive in detecting potential differences in efficacy than trials using hard clinical end-points.

However, PD markers may also be used as end-points in clinical efficacy studies in patients.

Examples of appropriate markers include haemoglobin for measuring the efficacy of an epoetin, and lactate dehydrogenase (which is a sensitive biochemical marker of intravascular haemolysis) for evaluating the efficacy of a complex drug such as eculizumab. For denosumab, investigation of bone formation and resorption markers as part of the PK study may be useful or possibly sufficient. This would involve measurement of bone mineral density and bone turnover markers such as serum C-terminal telopeptide of type 1 collagen (CTX-1) and procollagen type 1 N-terminal propeptide (P1NP) after denosumab administration.

In certain cases (for example, when analytical similarity of the active ingredient in the biosimilar and the RP can be demonstrated to such a degree that clinical differences can be excluded) a comparative PK study may provide sufficient clinical evidence to support biosimilarity. However, a risk assessment

(including for example, the impurity profile) should be conducted to determine the need for additional safety/immunogenicity data on the biosimilar (see sections 9.5 and 9.6 below).

9.4 Efficacy studies

A comparative efficacy trial may not be necessary if sufficient evidence of biosimilarity can be inferred from other parts of the comparability exercise. A comparative clinical trial, if necessary, should confirm that the clinical performance of the biosimilar and the RP is comparable. Demonstration of comparable potency, PK and/or PD profiles provide the basis for use of the RP posology in the comparative clinical trial.

If a comparative clinical trial of the biosimilar and RP is deemed necessary then it is expected that it will be an adequately powered, randomized and controlled clinical trial performed in a patient population that allows for sensitive measurement of the intended clinical parameters. The principles of such trials are laid down in relevant ICH guidelines (*26*–*28*).

In principle, equivalence trial designs (requiring lower and upper comparability margins) are preferred for comparing the efficacy and safety of the biosimilar and RP. Non-inferiority designs (requiring only one margin) (*26*) or trials with asymmetrical margins may be considered if appropriately justified (*29*). Regardless of which design is selected in a particular case, the comparability margin(s) must be pre-specified and justified on the basis of clinical relevance – that is, the selected margin should represent the largest difference in efficacy that would not matter in clinical practice. Treatment differences within this margin would therefore be acceptable as they would have no clinical relevance.

Similar efficacy implies that similar treatment effects can be achieved when using the same posology, and the same dosage(s) and treatment schedule should be used in clinical trials comparing the biosimilar and RP. In this regard, equivalence trials are again preferable to ensure that the biosimilar is not clinically less or more effective than the RP when used at the same dosage(s).

A non-inferiority design could be acceptable, if justified by the applicant, for example:

- for biological products with high efficacy (for example, a response rate of over 90%), making it difficult to set an upper margin; or
- in the presence of a wide safety margin.

When using asymmetrical margins, the narrower limit should rule out inferior efficacy and the broader limit should rule out superior efficacy. The use of asymmetrical margins should be fully justified by the sponsor of the

proposed biosimilar. Factors that would allow for the use of such margins in a clinical trial include:

- if the dose used in the clinical study is near the plateau of the dose–response curve; and
- there is little likelihood of dose-related adverse effects (for example, toxicity).

The final results obtained from the comparative clinical trial(s) along with comparative analytical, functional and PK data will determine whether the biosimilar and the RP can be considered to be clinically similar. If clinically relevant differences are found, a root cause analysis should be performed. If a plausible cause that is unrelated to the product (for example, inadvertent baseline differences between treatment groups despite randomization) cannot be found, the new product should not be considered to be similar to the RP.

Careful consideration should be given to the design of the comparative study/studies, including the choice of primary efficacy end-point(s). Studies should be conducted using a clinically relevant and sensitive end-point within an homogenous population that responds well to the pharmacological effects of the biological product of interest to show that there are no clinically meaningful differences between the biosimilar and RP. Clinical outcomes, surrogate outcomes (PD markers) or a combination of both can be used as primary end-points in biosimilar trials. The same study end-points used to establish the efficacy of the RP may be used because a large body of historical data would generally be available in the public domain for setting the comparability margin(s) and calculating the sample size. However, the primary end-point could be different from the original study end-point for the RP if it is well justified and relevant data are available to support its use as a sensitive end-point and its suitability for the determination of the comparability margin(s). A relevant PD end-point can be used as the primary end-point – for example, when it is a known surrogate of efficacy or when it can be linked to the mechanism of action of the product. The primary or secondary end-points can also be analyzed at different time points compared to those used in clinical trials with the RP if these are considered to be more sensitive in capturing the pharmacological action(s) of the biological product – for example, adalimumab efficacy could be measured by responses at week 12 or 16 in addition to week 24.

The sample size and duration of the comparative clinical study should both be adequate to allow for the detection of clinically meaningful differences between the biosimilar and RP. When a comparative clinical trial is determined to be necessary then adequate scientific justification for the choice of study design, study population, study end-point(s), estimated effect size for the RP and comparability margin(s) should be provided and may be discussed with regulators in order to obtain agreement at least in principle prior to trial initiation.

9.5 Safety

Safety data should be captured throughout clinical development from PK/PD studies and also in clinical efficacy trials when conducted. Knowledge of: (a) the type, frequency and severity of adverse events/reactions when compared with the RP; (b) whether these are due to exaggerated pharmacological actions; (c) the degree of analytical and functional similarity of the biosimilar and RP; and (d) the presence of novel impurities and novel excipients in the biosimilar will all inform the type and extent of data required to characterize the safety profile of the biosimilar.

If the clinical programme for the biosimilar is limited to confirmatory PK/PD studies, this will need to be adequately justified and a risk assessment should be conducted to determine the need to obtain additional safety data for the biosimilar. For example, for insulin the most relevant safety issue is hypoglycaemia which can be attributed to its pharmacological action. Highly similar physicochemical characteristics and PK/PD profiles of the biosimilar and RP could provide sufficient reassurance that the risk of hypoglycaemia is also similar, obviating the need for further safety data. Similar examples are teriparatide, filgrastim or somatropin. The current data suggest that more-complex products such as mAbs can be sufficiently characterized and also fall into this category (*22*).

If the biosimilar contains impurities that are not present in the RP (for example, because of the use of a novel expression system) then the generation of further safety data may be necessary, or scientific justification should be provided as to why such data are not needed. Manufacturers should consult with regulators when proposing a clinical programme solely relying on PK/PD studies.

As for all medicinal products, further monitoring of the safety of the biosimilar will be necessary in the post-marketing phase (see section 10 below).

9.6 Immunogenicity

Immunogenicity should be investigated as part of the clinical evaluation package of the biosimilar relative to the RP unless the manufacturer can provide a scientific justification that human immunogenicity data are not needed. Such justification should be based on the degree of physicochemical similarity of the biosimilar and RP, and on a thorough risk assessment of any unwanted immunogenicity and clinical consequences known for the RP. Although published information will be useful in gaining knowledge of the immunogenicity risk of the RP and in planning the immunogenicity strategy, it is not generally sufficient to support approval of the biosimilar. The goal of the immunogenicity programme is to exclude an unacceptable/marked increase in the immunogenicity of the biosimilar when compared with the immunogenicity of the RP and to generate descriptive data in support of biosimilar approval and its clinical use. If conducted,

the immunogenicity study report should include data on antibody incidence, magnitude of ADA response and neutralization ability, whether antibodies are transient or persistent, and their impact on PK and clinical correlates (*30*).

The marketing authorization application should include an integrated immunogenicity summary comprising a risk assessment and, if appropriate, the results of testing using appropriately validated and characterized assays, along with details on the clinical study duration, sampling schedules and regimen, and the clinical immunogenicity assessment (*30–32*).

The immunogenicity studies should be tailored to each product and require a multidisciplinary approach taking into account both quality and clinical considerations. The risk assessment should include:

- accumulated information on the immunogenicity of the RP (that is, on the nature, frequency and clinical relevance of the immune response);
- consideration of the quality aspects (including the nature and complexity of the drug substance, non-glycosylated/glycosylated, expression system, product- and process-related impurities, and aggregates);
- consideration of excipients and container closure system, and stability of the product, route of administration, dosing regimen; and
- consideration of patient- and disease-related factors (for example, immune competent/compromised and any concomitant immunomodulatory therapy).

Placing particular emphasis on any differences in product-related factors (for example, impurities arising from a novel expression system and/or novel excipients) that could modify immunogenicity will be crucial in the risk assessment of the biosimilar. Importantly, consideration of the type of product is also a critical element of the risk assessment, with the risk being higher for a product that has an endogenous non-redundant counterpart (for example, epoetin). In such cases, special attention should be paid to the possibility of the immune response seriously affecting the endogenous protein and its unique biological function, with serious adverse effects. Real-time testing for neutralizing ADAs is recommended for epoetins (*33*) and other high-risk products (for example, enzyme replacement therapies and coagulation factors). Conversely, for well-characterized biological substances (for example, insulin, somatropin, filgrastim, teriparatide), where an extensive literature and clinical experience indicate that immunogenicity does not impact upon product safety and efficacy, immunogenicity studies may not be necessary, provided that the biosimilar is highly similar to the RP and the risk-based evaluation indicates a

low risk. This may also be applicable to other products, including mAbs. In such cases, manufacturers should consult with the regulatory authorities.

Appropriate scientific justification for not conducting a safety/immunogenicity study should always be provided.

9.6.1 Immunogenicity testing

A multi-tiered approach comprising screening and confirmatory immunoassays that detect binding ADAs followed by assays which determine ADA magnitude and neutralization potential is generally necessary and deviation from this requires justification.

Information on current assays and formats and on their benefits and limitations, along with the interpretation of results, has been extensively reviewed (*33–36*). The manufacturer will need to justify the antibody-testing strategy and the choice of assays to be used. Attention should be given to the selection of suitable controls for assay validation and to the determination of cut-off points for distinguishing antibody-positive from antibody-negative samples. Aspects relating to potential interference by matrix components, including the pharmacological target and the residual drug in the sample, are also important. To mitigate such interference, corrective measures should be implemented. For example, for drug interference (which commonly occurs with samples taken from patients given mAbs) measures such as allowing time for clearance of the drug from the circulation prior to sampling, or incorporating steps for dissociating immune complexes and/or removal of the drug can be used. Care should be taken to ensure that the use of such measures does not compromise ADA detection or patient treatment.

Where required, comparative immunogenicity testing should be performed using the same assay format and sampling schedule. For immunogenicity assessment in new drug development, antibody testing is performed using the therapeutic given to the patient. In applying this concept to biosimilars, the development of screening assays with a similar sensitivity for the two patient groups (biosimilar and RP) within the same study is very challenging. Therefore, in the biosimilar scenario, relative immunogenicity is often assessed by using a single assay which employs the drug substance of the biosimilar as the antigen for sample testing for both groups. This approach allows for the detection of all antibodies developed against the biosimilar. The manufacturer should demonstrate the suitability of the method(s) used and provide data assuring that the method(s) measure ADA to the RP and to the biosimilar to a similar extent (*25*).

Neutralization assays reflecting the mechanism of action are usually based on the potency assay of the product. Non-cell ligand-based assays are relevant in cases where the therapeutic binds to a soluble ligand and inhibits its biological action. For products associated with high risk (for example, those with

non-redundant endogenous homologs) and those for which effector functions are important, the use of functional cell-based bioassays is recommended. Where necessary, advice on the need for a neutralization assay and on the appropriate format to use (cell-based, ligand-based or based on enzyme activity) may be sought from regulatory authorities.

Further characterization of antibodies (for example, isotype) should be conducted if considered clinically relevant, or in special situations (for example, the occurrence of anaphylaxis or use of certain assay formats), taking into account the immunogenicity profile of the RP. For example, if the RP does not elicit an IgE response it is unlikely that the biosimilar would elicit one if the same expression system is used. The retention of patient samples under appropriate storage conditions will be necessary for retesting in cases where technical problems occurred with the original assay.

9.6.2 Clinical evaluation

ADAs can affect the PK, PD, safety and/or efficacy of the administered product. The immunogenic risk of a biological is determined by the ADA incidence in the treated population and the magnitude of the unwanted clinical effect, and influences the benefit–risk balance of the therapeutic.

If human immunogenicity data are needed, they should be generated in a comparative manner throughout the clinical programme. The sensitive patient population (that is, the population with the highest likelihood of mounting an immune response) is preferred for investigating immunogenicity. For example, if an epoetin is licensed for the treatment of renal anaemia and for patients with chemotherapy-induced anaemia, the selection of patients with renal anaemia is advised. Comparative PK and/or PD studies should be designed to also collect immunogenicity data regardless of the population to be included (for example, healthy volunteers and patients). A PK/PD cross-over design is possible for immunogenicity testing but if the exposure time until the switch does not provide sufficient immunogenicity data, the sponsor must ensure that a sufficient number of patients are treated without cross-over – for example, by extending the cross-over study with two parallel treatment arms, or by proposing a separate immunogenicity study.

If ADAs are known to affect the PK of the RP then ADA rate and kinetics assessments could be performed along with assessment of their impact on PK through pre-specified subgroup analysis of ADA-negative and -positive subjects.

The observation period required for immunogenicity testing will depend on the expected time of antibody development and should be justified by the manufacturer. Sampling during immunogenicity testing should include baseline sampling (prior to treatment) for pre-existing antibodies, sampling during treatment and in some cases post-treatment, particularly if ADAs persist or are

undetectable at earlier time points (due to immunosuppressive properties of the product or technical problems such as drug interference). The sampling schedule should be synchronized for evaluation of PK as well as for assessment of safety and efficacy to provide an understanding of the impact of antibodies on clinical outcome. Generally, for chronic administration, 6-month data are acceptable to exclude excessive immunogenicity, but in some cases a longer evaluation period may be appropriate pre-licensing to assess antibody incidence and possible clinical effects.

Furthermore, notable differences in immunogenicity between the biosimilar and RP would require further investigation of the underlying cause, and data and justification provided to support any claim that the difference noted was not clinically relevant. An analysis of the clinical impact of ADAs in both arms on PK, efficacy and/or safety should be performed through stratified analysis of ADA-negative and -positive subjects.

Any potential for the production of neutralizing antibodies against critical endogenous factors (for example, following epoetin administration) will necessitate clinical studies in patients.

As is the case with the RP, the biosimilar should also undergo robust post-marketing surveillance that includes assessment of any serious adverse events related to immunogenicity.

9.7 Authorization of indications

The decision to authorize the requested indications will be dependent upon the demonstration of similarity between the biosimilar and RP. The extension of indications from the RP to the biosimilar is only possible if the following requirements are fulfilled:

- similarity in analytical characteristics and functional properties has been confirmed in sensitive orthogonal assays which provide information on the clinically relevant mechanism of action and/or involved receptor(s) as part of the comparability exercise; and
- this is supported by clinical data (comparative PK and/or PD study – see sections 9.1–9.3 above) plus a comparative clinical trial performed in a patient population that allows sensitive measurement of the intended clinical parameters, if necessary (see sections 9.4–9.6 above).

For example, authorization of all indications may be obtained based on highly comparable functional data – for example, for biosimilars of mAbs such as infliximab and adalimumab if they show fully comparable activity (including ADCC, CDC, reverse signalling and apoptosis) both in terms of binding to soluble TNF and membranous TNF.

10. Pharmacovigilance

Following approval, many NRAs consider a biosimilar to have its own life-cycle and there is no formal requirement to re-establish similarity to the RP when comparability exercises are conducted following manufacturing changes (*9*, *17*). Both RP and biosimilar manufacturers are responsible for ensuring that their products remain safe and efficacious throughout their life-cycle by preventing significant changes to individual products. In this context, it is important to emphasize that the required data can be obtained only by having robust pharmacovigilance systems in place that allow for the collection of product-specific data.

As with all medicinal products, further close monitoring of the safety and efficacy of a biosimilar in all approved indications, along with continued benefit–risk assessment, are necessary in the post-marketing phase. Any specific safety monitoring or risk-minimization measures imposed on the RP or product class should be incorporated into the pharmacovigilance plan for the relevant biosimilar unless a compelling justification can be provided to show that this is not necessary. Furthermore, participation in existing disease registries should be encouraged and is mandatory if also mandatory for the RP. Post-marketing safety reports should include all information on product safety received by the marketing authorization holder. The safety information must be evaluated in a scientific manner and this should include evaluation of the frequency and cause of adverse events.

The manufacturer should submit a pharmacovigilance plan describing a safety specification, pharmacovigilance activities and risk-minimization activities at the time of submission of the marketing authorization application or whenever a safety concern arises post-marketing. The principles of pharmacovigilance planning can be found in relevant guidelines such as ICH E2E (*37*). The safety specification should describe important identified or potential safety issues for the RP and for the substance class as well as any that are specific to the biosimilar. If there are any remaining uncertainties regarding the biosimilar – due for example to the use of a novel excipient or device – then these should be included in the pharmacovigilance plan and followed up post-marketing.

Manufacturers should ensure that at the time of the marketing authorization they have in place an appropriate pharmacovigilance system, including the services of a qualified person responsible for monitoring pharmacovigilance activities and the necessary means for notification of adverse reactions that occur in any of the countries in which the product is marketed.

After the marketing authorization has been granted, it is the responsibility of the NRA to monitor closely the compliance of manufacturers with their marketing commitments, particularly with regard to their pharmacovigilance obligations as described here.

In addition, as with all biological products, an adequate system for ensuring the specific identification of the biosimilar (that is, traceability) is essential. The NRA shall provide a legal framework for proper pharmacovigilance surveillance and ensure the ability to identify any biological marketed in its area of jurisdiction that is the subject of an adverse reaction report. In addition to the international nonproprietary name (INN) (*38*) an adverse reaction report for any biological should also include all other important indicators, including the proprietary (brand) name, manufacturer's name and lot number. The country of origin is not strictly required.

11. Labelling and prescribing information

The biosimilar should be clearly identifiable by a unique trade name together with the INN. From the perspective of WHO there is no specific INN nomenclature for biosimilars – that is, there is no part of an INN which indicates that a product is a biosimilar. Biosimilars are assigned INNs using the process and rules used for all biological products. In many cases, the INN for a biosimilar is the same as that for its RP – for example, for G-CSF biosimilars that have used Neupogen as the RP, both the biosimilar and the RP have the INN "filgrastim" (*39*, *40*). Provision of the lot number is essential as it is an important part of production information and is critical for traceability whenever problems with a product are encountered.

The prescribing information for a biosimilar should be as similar as possible to that of the RP except for product-specific aspects such as use of different excipient(s) and/or presentations. This similarity is particularly important for posology and for safety-related information, including contraindications, warnings and known adverse events. However, if there are fewer indications for the biosimilar than for the RP, the related text in various sections may be omitted unless it is considered important in informing doctors and patients of certain risks – for example, as a result of potential off-label use. In such cases it should be clearly stated in the prescribing information that the biosimilar is not intended for use in the specific indication(s) and the reasons why.

12. Roles and responsibilities of NRAs

One of the responsibilities of an NRA is to set up appropriate regulatory oversight for the licensing and post-marketing surveillance of biosimilars that are developed and/or authorized for use in its area of jurisdiction. The experience and expertise of the NRA in evaluating biological products is a key prerequisite for appropriate regulatory oversight of these products. The NRA is responsible for clearly defining a suitable regulatory framework for licensing biological products, including biosimilars (*41*).

As the development of biological products is a rapidly evolving area, NRAs may need to conduct regular reviews of their licensing, the adequacy of their regulations for providing oversight, and the processes and policies that constitute the regulatory framework. Such a process of review is an essential component of well-functioning and up-to-date regulatory oversight of biological products (*42*). Some countries have licensed products called "biosimilars" that were approved prior to the establishment of a regulatory framework for biosimilar approval. WHO recommends avoiding use of this term (or other equivalent term) for products that have not been evaluated in line with the principles set out in these Guidelines. NRAs should develop a specific, appropriate, regulatory framework for approving biosimilars that is distinct from the regulatory procedures previously applied to products with a version of the same active ingredient intended for the same use but for which regulatory evaluation was not well defined (*41*, *43*). In addition, the terminology used for such products should not be confused by calling them "biosimilars".

NRAs could improve access to biosimilars of assured quality, safety and efficacy by improving the efficiency of their regulatory evaluation – for example, by making efforts to reduce the time taken for evaluation without compromising the quality of the review process (*41*, *43*). In addition, efforts should be made to avoid the unnecessary duplication of studies (*44*).

Most countries either use or amend their existing legislation and applicable regulations or develop entirely novel regulatory frameworks for the authorization of biosimilars. In some jurisdictions, regulations for licensing subsequent entry versions of biotherapeutic products are intricately linked with policies for innovation. Hence an NRA may need to coordinate and communicate with other stakeholders to ensure consistency (*45*).

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Annex 4

Guidelines for the production and quality control of monoclonal antibodies and related products intended for medicinal use

Replacement of Annex 3 of WHO Technical Report Series, No. 822

Guidelines published by the World Health Organization (WHO) are intended to be scientific and advisory in nature. Each of the following sections constitutes guidance for national regulatory authorities (NRAs) and for manufacturers of biological products. If an NRA so desires, these WHO Guidelines may be adopted as definitive national requirements, or modifications may be justified and made by the NRA. It is recommended that modifications to these Guidelines are made only on condition that such modifications ensure that the product is at least as safe and efficacious as that prepared in accordance with the guidance set out below.

Abbreviations

ADCC	antibody-dependent cell-mediated cytotoxicity
ADCP	antibody-dependent cellular phagocytosis
C1q	complement component 1q
CDC	complement-dependent cytotoxicity
CDR	complementarity-determining region
CHO	Chinese hamster ovary
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunosorbent assay
EU	Endotoxin Unit(s)
Fab	fragment antigen-binding (region)
Fc	fragment crystallizable (region)
Fv	variable fragment(s)
GMP	good manufacturing practices
hcDNA	host cell DNA
HCP	host cell protein
HPLC	high-performance liquid chromatography
HVAC	heating, ventilation and air conditioning
ICH	International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
LAL	*Limulus* amoebocyte lysate (test)
mAb	monoclonal antibody
MCB	master cell bank
mRNA	messenger RNA
MSB	master seed bank
NCL	national control laboratory
NRA	national regulatory authority
PCR	polymerase chain reaction

PEG	polyethylene glycol
PPQ	process performance qualification
QbD	Quality by Design
rDNA	recombinant DNA
scFv	single-chain variable fragment(s)
SEC	size-exclusion chromatography
SPF	specific-pathogen-free
SPR	surface plasmon resonance
TSE	transmissible spongiform encephalopathy
WCB	working cell bank
WSB	working seed bank

Introduction

The WHO Guidelines for assuring the quality of monoclonal antibodies for use in humans (*1*) were adopted on the recommendation of the WHO Expert Committee on Biological Standardization at its forty-second meeting in October 1991. Since that time there have been extensive technological advances in the manufacture and quality assurance of monoclonal antibodies (mAbs) – most notably involving the use of recombinant DNA (rDNA) and cloning technologies.

In 2013, the WHO Guidelines on the quality, safety and efficacy of biotherapeutic protein products prepared by recombinant DNA technology (*2*) were also adopted on the advice of the Committee. Although guidance on the manufacture and quality control of mAbs is within the scope of these Guidelines, requests were subsequently made to WHO to provide additional clarity and more specific details unique to this subject, as well guidance on mAbs manufactured using plant-based systems.

As a result, the present Guidelines were developed through a process of international consultation and are intended to replace the 1991 Guidelines above.

Purpose and scope

Although these Guidelines are primarily intended to provide guidance on regulatory considerations and requirements for marketing authorization purposes, reference is also made throughout the document to products within their clinical development programmes. Such guidance is provided to highlight issues which may be important to consider during the product development process.

Following significant increases in the types and varieties of mAbs, and related antibody-like proteins, these Guidelines can be considered to be applicable to those that are based on an antibody framework, including:

- mAbs of all isotypes, whether they are humanized, human, or chimeric, and regardless of the intended therapeutic mechanism of action;
- antibody fragments, such as single-chain variable fragments (scFv), and fragment antigen-binding (Fab) and fragment crystallizable (Fc) regions;
- single domain antibodies;
- bispecific or multispecific antibodies;
- Fc-fusion proteins;

- mAbs or related antibody proteins that have been chemically modified, such as through conjugation to polyethylene glycol (PEG) or an active drug substance; and
- multiple mAb substances co-formulated within a final product ("antibody cocktail").

For the purposes of this document, the term "monoclonal antibody (mAb)" encompasses the range of substances and products listed above unless otherwise stated. For additional guidance on the characterization and quality assessment of relevant biosimilar products, the WHO Guidelines on evaluation of monoclonal antibodies as similar biotherapeutic products (SBPs) should be consulted (*3*).

It should be noted that the current document is not applicable to nucleic-acid-based platforms which use a vector or similar technology for delivery of the genetic sequence that encodes for antibody production in vivo following administration. However, for mAbs produced in vivo following the administration of messenger RNA (mRNA), some aspects discussed in section 6 of the WHO regulatory considerations document on evaluating the quality, safety and efficacy of messenger RNA vaccines for the prevention of infectious diseases (*4*) may be applicable as the manufacturing steps of such products may be similar.

The production of antibody mimetic proteins based on non-immunoglobulin scaffolds (for example, DARPins, affimers, and anticalins) may involve manufacturing and quality assurance processes similar to those described in the current Guidelines. However, they may also require unique regulatory considerations which are outside of the scope of this document. Therefore, manufacturers of such products are encouraged to refer instead to the above-noted WHO Guidelines on the quality, safety and efficacy of biotherapeutic protein products prepared by recombinant DNA technology (*2*).

Although many mAb products are intended for parenteral administration, several are under development for administration via other routes (for example, intranasal, inhaled, oral, intravesical or intrathecal). The manufacture and control of mAbs should be the same regardless of the intended route of administration. However, some specifications (such as those for endotoxin, bioburden, sterility or purity) may have different stringency requirements compared to parenterally administered products. Similarly, the selection of excipients may include those which are not suitable for parenteral administration.

The current document also provides some guidance specific to mAbs manufactured using plant-based systems. However, the general principles provided will also apply to other novel expression systems, such as cell-free systems or transgenic animals, particularly from the purification steps onward. Appendix 1 provides examples of expression systems currently used in the

production of mAbs for development or marketing purposes. Although these Guidelines include guidance on the production of mAbs and related proteins in animal systems (for example, from mouse ascites), WHO strongly discourages the use of in vivo production methods for the manufacture of such human therapeutic products.

Terminology

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

Adventitious agents: contaminating microorganisms that can include bacteria, fungi, mycoplasmas, and endogenous and exogenous viruses, and that have been unintentionally introduced into the manufacturing process.

Antibody fragments: proteins that consist of regions, or sections, of antibody molecules. These are usually single-chain variable fragments (scFv), fragment antigen-binding (Fab) regions or single domain antibodies.

Biological activity: the ability or capacity of a mAb substance or product to elicit a defined biological effect in vitro (for example, in cultured cells or viruses) or in vivo (in animal models and/or humans).

Bispecific or multispecific antibodies: a single mAb in which each binding domain recognizes different epitopes of the same antigen or different antigens.

Co-formulated mAbs: a final product formulated to contain two or more mAbs, mAb conjugates and/or mAb fragments each of which recognizes a different epitope or antigen. These may also be referred to as "antibody cocktails", "antibody mixtures", "pooled antibody products" or "oligoclonal products". Co-formulated mAbs are not the same as individual mAb products which may later be co-administered during treatment.

Contaminants: materials inadvertently introduced to the mAb substance and/or product that are not intended to be part of the manufacturing process (for example, **adventitious agents**, microbial contaminants and endotoxin).

Drug product: a final product in a defined container closure system that contains one or more **drug substances** and which may be formulated with excipients.

Drug substance: the active pharmaceutical ingredient and associated molecules that may subsequently be formulated with excipients to produce the **drug product**.

Final bulk: a formulated preparation from which the final containers are filled. The final bulk is prepared from one or more purified mAb substances, formulated to contain all excipients and homogenous with respect to its composition.

Impurities: materials present in the mAb substance or product which are either: (a) product-related (for example, mAb molecular variants, aggregates or

fragments) and which do not have properties comparable to the desired product with respect to its safety, activity and efficacy; or (b) process-related (for example, reagents, media components, host cell proteins (HCPs) or leachates) and not considered to be the active ingredient.

Intermediate: a material produced during the production of an active pharmaceutical ingredient or **drug substance** that undergoes further molecular change or purification before it becomes the active pharmaceutical ingredient or drug substance.

Master cell bank (MCB): an aliquot of a single pool of cells which generally has been clonally derived under defined conditions, dispensed into multiple containers and stored under defined conditions.

Master seed bank (MSB): seeds of a selected transgenic plant from which all future production will be derived, either directly or through the production of a **working seed bank**.

Parental cell line: the clonally derived cell line produced or acquired by the manufacturer and on which the production of the MCB is based. The history of the parental cell line should be recorded whenever possible.

Platform technology: an existing technology, or group of technologies, applied to the development and/or production of similar mAb products by a manufacturer. A given manufacturer might have one or more platforms on which they will develop various mAbs. A platform would be considered when the elements of the manufacturing methods and/or processes, the mAb protein scaffold and compliance with good manufacturing practices (GMP) are essentially unchanged. The experience and knowledge gained, data generated (on manufacturing, control and stability) and the method validation can all be used as supportive data for the more rapid assessment and development of a new candidate mAb product that fits within the boundaries of the platform.

Recombinant DNA technology: technology that joins (that is, recombines) DNA segments from two or more different DNA molecules that are then inserted into a host organism to produce new genetic combinations. It is also referred to as gene manipulation, gene editing or genetic engineering because the original gene is artificially altered. These new genes, when inserted into the expression system, form the basis for the production of rDNA-derived protein(s) (*2*).

Source material/starting material: any substance of a defined quality used in the production of a biological product, excluding packaging materials.

Working cell bank (WCB): a cell bank produced by expansion of the clonally-derived **MCB** under defined culture conditions.

Working seed bank (WSB): a seed bank derived by the propagation of seeds from an **MSB** under defined conditions and used to initiate production seed cultures on a lot-by-lot basis.

General considerations

Monoclonal antibodies (mAbs) are immunoglobulins derived from a monoclonal cell line that have a defined specificity. Their immunological activities are based on binding to a specific ligand or antigen and may depend on other effector functions. Encompassing a wide range of clinical indications, mAbs represent a large class of therapeutic biological products that continue to transform modern medicine. In recent years, such products have dominated the biotherapeutics market, with hundreds of novel mAbs and mAb-like proteins now in clinical development (*5*). The success of therapeutic mAbs can largely be attributed to their specificity and the technological advances that have driven their development. However, with multiple functional domains within a single molecule, mAbs are structurally and functionally complex proteins. This has implications for their production and quality control, and hence for the way in which they are regulated.

Antibody development

Historically, murine hybridoma technology, developed in the 1970s, paved the way for modern approaches to mAb discovery, and continues to be used as the basis for generating chimeric and humanized mAbs using recombinant DNA methods. The development of phage display techniques in the 1990s provided a powerful approach to screening for peptides or antibody fragments (scFv or Fab) specific for therapeutic targets (*6*). Phage display technology has also been used to emulate antibody maturation, combining site-directed mutagenesis of complementarity-determining region (CDR) sequences with iterative cycles of affinity selection (*7*). Transgenic mice expressing only human immunoglobulin genes also provide an effective alternative for the identification of fully humanized antibodies. More recently, bispecific antibodies with novel functions have been engineered by linking antigen-binding domains (such as scFv or Fab) with different specificities within the same antibody molecule. In addition, innovative products have been developed in which mAbs or antibody fragments have been conjugated to small-molecule drugs, capitalizing on their specificity to target such drugs to particular sites or tissues.

Ongoing improvements in antibody engineering combined with greater knowledge of their immunomodulatory properties continue to give rise to new and improved products for the treatment of an increasing range of human diseases – each with specific target antigen(s) and mechanisms of action. Regardless of the process behind the development of the drug substance, the structure of the mAb is critical to the immunological and effector properties of the product. Regulatory assessment should be based on careful consideration of the rationale for the suitability of the mAb for its intended indication, including the choice of its specific target(s), the affinity of the antibody for that target, product half-life and its mode of action. This will require a thorough understanding of the role of

the target in the development of disease and of the way in which the mAb exerts its biological effect – for example, blocking the binding of a ligand or infectious agent to a receptor, or mediating cytotoxicity via its Fc region.

To ensure product safety and efficacy, the risk of it eliciting antidrug antibody responses in patients should be carefully considered, particularly if the structure or post-translational modification of the mAb differs from natural human immunoglobulin. Similarly, care should be taken to ensure that the formulated product (which will include excipients and/or matrix components in addition to the mAb) does not induce hypersensitivity, autoimmunity or other adverse reactions in recipients.

The use of a platform technology that allows a manufacturer to rapidly develop and produce multiple mAb products based on a common scaffold structure and manufacturing processes which are essentially unchanged poses unique regulatory considerations. Experience gained from the development of one product may be applied in the development of another. Subject to the demonstration of comparability between the manufacturing processes used for each mAb product, the knowledge and data from the manufacturing process of one product may support the development of the manufacturing process of another.

Cell substrate and mAb production

The cell line chosen for mAb production must be stable in culture and should be considered in terms of its ability to consistently produce a biologically active protein of the desired quality. If the cell substrate is genetically engineered, the expression system should be described in accordance with the relevant WHO and ICH guidance. If cell fusion or transformation is used to immortalize B lymphocytes for use in mAb production, the safety of the approach should be carefully considered. Where human B lymphocytes are the parental cell line, careful consideration should be given to the possibility of contamination with a defective prion or other pathogenic adventitious agents (see section A.4.2.3 below).

The culture medium and growing conditions used will have a direct impact on cell growth, the amount of mAb produced and product quality. Mammalian cell culture media are inherently complex and historically have included animal sera in their composition to meet the nutritional requirements of the cells. To reduce the risk from adventitious agents (such as the prion responsible for transmissible spongiform encephalopathies (TSEs) or viruses of animal origin) well-defined media have been developed that are free from animal material and suitable for a range of cell substrates, including Chinese hamster ovary (CHO) and NS0 cells. The use of such chemically defined production media has several advantages, including improving consistency between production lots, providing greater control over production, and facilitating downstream purification and quality control processes. Mammalian cell culture conditions should be well defined, with temperature, pH,

and dissolved oxygen and carbon dioxide levels monitored along with any other identified critical parameters. Different cell culture systems (for example, fed-batch, perfusion or continuous) may be used to favour cell growth and/or mAb production to achieve the required cell density and mAb production level.

Downstream processing

Following the growth and production stages, the mAb should be recovered from the cell substrate using a process that consistently delivers a mAb substance suitable for its intended use – however, the specific details of downstream processes and their controls will be unique to each product and manufacturer. Typically, the first step of this process involves the removal of cells and cell debris. This initial purification may be achieved using a combination of centrifugation, depth filtration and membrane filtration. Most mAb purification processes then involve protein A- or protein G-based chromatography to capture the mAb, with any remaining process and/or product-related impurities removed through subsequent rounds of chromatography. Finally, the mAb substance may be concentrated and/or diafiltered into formulation buffer followed by filling, storage and shipping.

Consideration should be given to the source of the protein A- or protein G-containing chromatography media and their method of preparation to ensure a low risk of contamination with adventitious agents. Depending on the production system, downstream processing should also consider any requirements for viral safety, and include viral clearance purification steps as appropriate.

Because of the structural similarity of different mAb products, the knowledge and technological experience of the manufacturer may be used to develop platform manufacturing processes which may be applicable to related mAb products. These might include the cell culture system, expression vectors, purification schemes, container systems and analytical methods. Experience gained in assuring the quality of one product can provide supporting insights into assuring the quality of other mAb products made using the same technology and process, but recognizing different antigens. Nevertheless, manufacturing processes based on a platform manufacturing approach should be validated for a specific commercial product and production site. Given that quality attributes are process and product specific, the control strategy will also be product specific, and the appropriateness of analytical methods developed for other products using the same platform approach will require careful consideration.

Quality by Design

Quality by Design (QbD) is a systematic approach to product development that utilizes detailed knowledge of a product, the processes employed in its manufacture and the associated process controls to ensure consistent product quality, safety and efficacy. The underlying principles of this approach are

set out in ICH guidelines Q8–Q11 (*8*–*11*) and apply throughout the life-cycle management of a mAb product. Extensive knowledge of antibody structure and function, together with ever-increasing experience of biopharmaceutical protein manufacturing processes, makes the application of QbD an attractive option in the development of innovative therapeutic mAbs.

The quality control and assurance of mAb products is challenging – largely due to them being very large and highly complex proteins with significant post-translational modifications that may impact their stability, pharmacokinetics and dynamics, immune reactogenicity, safety and efficacy. As biotechnological products, mAbs are also likely to contain process- and product-related impurities that may affect their quality. Furthermore, culture conditions can greatly influence mAb structure, with both the purification processes and any genetic, post-translational or chemical modifications further adding to the challenge of producing a product of consistent quality. In light of this, it is important for the manufacturer to identify critical quality attributes of mAb substances and products early in their developmental stage and to understand the impact of process parameters on product quality.

Heterogeneity

Although mAbs are by definition characterized by a unique amino acid sequence, they are subject to post-translational modifications, as well as to physicochemical transformations that arise during their production and storage. In practice, the drug substance and the drug product usually include a low level of sequence variants that arise from the inherent errors that typically occur during transcription and translation. Such heterogeneity is specific to the manufacturing process, and its potential impact on the activity, efficacy, safety and pharmacokinetic properties of a mAb product should be understood in order to ensure batch-to-batch consistency. In addition, heterogeneity may affect both the long-term stability and immunogenicity of a therapeutic mAb – though, in general, modifications that are found in natural human antibodies are less likely to be immunogenic or pose a safety risk. The types of modifications commonly associated with therapeutic mAbs include N- and C-terminal modifications, glycosylation, glycation, disulphide bond formation, isoforms/variants and various other amino acid related modifications.

N-terminal pyroglutamate is a common modification of natural immunoglobulin G (IgG). However, relatively minor changes in manufacturing conditions (for example, buffer composition, pH and temperature) can result in variable levels of N-terminal pyroglutamate in therapeutic mAb products (*12*). Another common N-terminal modification associated with mAbs (rather than natural IgG) is the incomplete removal of signal peptides resulting in mAbs with signal peptides of variable size, contributing to heterogeneity in the mass of the product (*13*, *14*).

Generally, mAbs are synthesized with a C-terminal lysine on their heavy chain that is subsequently removed during mammalian cell culture by basic carboxypeptidase activity. Incomplete removal of lysine results in a product with variable levels of C-terminal lysine. Although C-terminal lysine does not affect mAb structure, stability or pharmacokinetic properties, its presence has been reported to interfere with complement component 1q (C1q) binding and complement-dependent cytotoxicity (CDC) (*15*). However, after administration, any remaining C-terminal lysine is rapidly removed (*16*). As heterogeneity caused by C-terminal lysine affects both mass and charge, it can be detected by mass spectrometry, isoelectric focusing or ion-exchange chromatography (*17*). In contrast to natural human IgGs (which have very low levels), C-terminal amidation has also been reported as a common modification contributing to the heterogeneity of recombinant IgG1 mAbs produced in CHO cells (*18*).

As with natural IgG molecules, mAbs have a conserved N-glycosylation site in the Fc region of IgG, which has a strong influence on antibody conformation, and is where certain glycan structures impact binding to Fcγ or high-mannose receptors. Based on its primary structure, the Fab region may also contain N-linked oligosaccharides which, depending on their location, may affect antigen binding. Antibodies that are aglycosylated tend to be destabilized, have a propensity to aggregate and may have altered receptor binding activity – all of which could have implications for their effector functions and immunogenicity (*19*). Aglycosylation contributes to mAb heterogeneity at low levels.

The glycosylation-related heterogeneity of mAbs is primarily associated with galactosylation, fucosylation, mannosylation and sialylation of the biantennary complex oligosaccharides – though the presence of other low-abundance oligosaccharides can also contribute to mAb heterogeneity. Such modifications have been demonstrated to influence the binding activities, immunological functions – for example, Fcγ receptor binding, C1q binding and/or antibody-dependent cell-mediated cytotoxicity (ADCC) – and pharmacokinetics of mAbs (*20–26*). However, changes to one property do not necessarily indicate changes to another property. Therefore, an assessment and understanding of mAb glycosylation and glycoform heterogeneity during product development is important – though the required extent of such characterization studies can depend on the mAb mechanism of action.

It should be noted that glycosylation patterns vary between species. Glycan species not naturally present in humans may occur in mAbs produced in nonhuman mammalian cell systems. For example, Galα1-3Gal is generated by some mammalian species but not human cells. These may pose a risk of induced immunogenic reactions. Cell culture conditions can also greatly influence glycosylation patterns. As such, changes in the glycosylation-related heterogeneity pattern may be a reflection of potential changes within the mAb production

system. For this reason, understanding the impact of culture conditions and raw materials on glycosylation can help in the selection of appropriate control strategies and ensure mAb production of consistent quality.

Glycation is the reaction between reducing sugars and the primary amines on the N-terminus or lysine side chains. It primarily occurs during antibody production because of the sugars present in cell culture media, but also to a lesser extent during storage or when administered in diluent containing sugars (*27*). Glycation causes heterogeneity both in molecular weight and charge, as well as increasing the propensity of the mAb to aggregate (*28*). The level of glycation of antibodies is generally low and usually has little or no biological effect – however, in some cases, the glycation of lysine in the CDRs can affect antigen binding (*29*). The impact of this type of glycation can be assessed during development of the product. Glycation appears to have little or no effect on Fc-related effector functions.

The well-established inter- and intra-chain disulphide bond arrangements between cysteine residues in IgG play a key role in the folding and structural stability of an antibody. Therefore, any heterogeneity arising from variation in the disulphide bonding pattern in recombinant mAbs warrants careful consideration because of its potential impact on antibody structure, stability and biological activity. A number of such variations have been identified, including alternative disulphide linkages, free sulphydryl groups, trisulphide bonding, formation of thioether, and cysteine racemization (*26*). Variants with non-classical disulphide bond arrangements, which arise from the formation of different inter-chain connections between cysteines in the Fab and hinge regions, have only been described in IgG2 and IgG4, and can occur in both recombinant mAbs and natural antibodies. While non-classical variants of IgG2 differ in biological activity compared with their classical counterpart, evidence suggests that non-classical variants of IgG4 only differ in stability (*30*). Free sulphydryl groups result from the incomplete formation of disulphide bonds in both recombinant and natural antibodies. They may also arise when an antibody contains an extra cysteine residue, typically in the CDR. The impact of free sulphydryl groups on biological activity or stability will differ from one product to another and should therefore be assessed for any given therapeutic mAb. The formation of a trisulphide bond from the interaction of an existing disulphide bond with hydrogen sulphide has been reported in some production systems. However, there is no evidence that trisulphide bonds affect either antigen binding or thermal stability. The decomposition of disulphide bonds back to cysteine residues, through a dehydroalanine and persulphide intermediate, followed by cross-linking of dehydroalanine and cysteine results in the formation of a non-reducible thioether bond. This reaction also accounts for the occurrence of D-cysteine residues in the disulphide bonds between heavy and light chains in both mAbs and natural human IgG (*31*).

Modifications to amino acid side chains are a major cause of heterogeneity observed in mAbs. Deamidation of asparagine and glutamine residues can occur at any stage during production and storage, depending on the mAb microenvironment (for example, buffer composition, pH and temperature). Residues in the CDRs are particularly susceptible to deamidation because of their flexibility and exposure to the medium. Isomerization of aspartate has also been observed in the CDRs of mAbs and, as with the deamidation of asparagine and glutamine, may impact antigen binding and potency. As a reaction intermediate of both asparagine deamidation and aspartate isomerization, succinimide is frequently found in CDRs and has also been shown to reduce potency (*32*). In addition, oxidation of several amino acids has been observed at low levels in natural human antibodies (*33*). In recombinant mAbs, methionine oxidation is often observed and when it occurs at the conserved residues in the Fc region causes conformational changes that negatively impact stability, CDC, binding to neonatal Fc receptor and in vivo half-life, while also increasing the possibility of aggregation (*34–36*). Tryptophan residues in CDRs are also sensitive to oxidation and this can have a negative impact on potency and stability, while also increasing the possibility of aggregation (*37*).

High molecular weight (aggregate) and low molecular weight (fragment) species are both examples of product-related impurities that contribute to the size heterogeneity of a mAb product. Size heterogeneity within a therapeutic mAb drug product can potentially compromise quality, safety and efficacy. Aggregation caused by a wide range of conditions may occur at any time during the manufacturing process or storage. The size and nature of the aggregate is typically dependent on the kind of stress that led to its formation. Aggregation can result in the loss of the therapeutic properties of the mAb and reveal new epitopes which may induce unintended immunity to the aggregate or the production of anti-drug antibodies in the patient. Given its potential pharmacological impact on therapeutic mAbs, aggregation warrants careful consideration during the production and control of the product. Similarly, fragmentation is a common type of degradation and may occur spontaneously or through the action of proteases released from the cells during culture. Trace elements and other media components may also impact the formation of low molecular weight species. Fragmentation may impact product potency and/or efficacy and, therefore, should be well characterized and controlled during the manufacturing process. As the fragmentation pattern can be unique to each substance and product, it may be applied as a critical element in assessing product stability, manufacturing consistency and, potentially, comparability.

Conjugation

The conjugation of small molecules, peptides or other proteins to mAbs offers considerable potential for the development of innovative biotherapeutics due to their ability to target and discriminate between diseased and healthy tissue.

Conjugation may also be used to provide more favourable pharmacokinetic and/or pharmacodynamic profiles to the mAb. Agents ("payloads") that may be conjugated to mAbs include cytotoxic molecules, radioisotopes, steroids, cytokines, mRNA and repeating units of PEG.

The mAb intermediate, the linker and the payload must each meet their own quality specifications prior to conjugation. The specifications for the mAb intermediate might not be the same as those for a non-conjugated mAb drug substance but should allow for efficient conjugation and yield a conjugated product with the desired structure, purity, post-translational modifications, binding properties, effector functions and/or other biological activities. It should be noted that the current document does not discuss the manufacture or quality control of the linker or the payload agent prior to their conjugation to the mAb and users should consult current regulations and guidelines appropriate to these substances. Similarly, the types of conjugation chemistries available for use are multiple and are not discussed in these Guidelines. The conjugation technology used will need careful consideration to ensure precise drug loading, and should be well defined and shown to yield a mAb conjugate of consistent structure and quality. In general, site-specific conjugation technologies result in a more homogeneous product and may offer better batch consistency than conjugation at random sites. However, such heterogenous conjugation systems may be acceptable – for example, for products with a low drug to antibody ratio.

Characterization

Given the challenges associated with manufacturing a product of consistent quality, it is important to have a robust strategy for characterizing the drug substance and product to ensure that critical quality attributes are maintained, and that the product meets its specifications. Such characterization would be expected to include assessment of physicochemical properties, biological activity, purity and impurities, and post-translational modifications – and should also take into consideration the relationship between the mAb structure and its functional activity.

Drug substance and product characterization typically includes methods for the determination of primary and higher-order structure. The amino acid sequence of the mAb can be deduced from its nucleotide sequence and confirmed by peptide mapping and mass spectrometry. Nucleotide sequences of master and working seeds or cells can be conveniently determined using high-throughput methods.

Generally, physicochemical techniques offer the necessary sensitivity for the analysis of antibody heterogeneity (Appendix 2). However, the risk of artefacts arising from certain sample-preparation methods must be taken into account. Variability in the N- and C-terminal amino acid residues can be

assessed using methods that detect their impact on the charge and mass of the mAb. As disulphide bonds between cysteine residues play a key role in antibody folding and structural stability, it is important to consider the presence of free sulphydryl groups and the integrity of disulphide bridges. In addition, because of the potential impact of glycosylation on antibody structure and function, the carbohydrate content and glycosylation profile should be determined, paying particular attention to the distribution of glycan structures and the level of mannosylation, galactosylation, fucosylation and sialylation.

Characterization of the biological activity of the mAb will include the use of binding assays to determine its specificity, affinity and avidity for the target epitope. Examples of analytical methods for evaluating binding include enzyme-linked immunosorbent assay (ELISA), surface plasmon resonance (SPR), bio-layer interferometry and isothermal titration calorimetry. The antigen used in these assays and its relevant epitope should be qualified for its intended purpose.

Characterization of the biological activity of the mAb should also include assessment of its ability to cause the desired effect, using appropriate assays such as those for Fc-effector function (for example, CDC, antibody-dependent cellular phagocytosis (ADCP) and ADCC), virus neutralization, anti-proliferation and cytotoxicity, or other cell-based assays reflective of the mechanism of action. It should be noted that the mechanism(s) of action of therapeutic mAbs may be species specific depending on the Fc region functions and/or the inter-species homology of the target antigen. The interaction of the Fc region of humanized mAbs with non-human Fc receptors may give misleading results, thus limiting the usefulness of in vivo assays in the characterization of biological activity.

Special considerations

After the initial clinical batches have been produced, mAb production, purification and other downstream processes may undergo considerable optimization. The process and product characterization should ensure the comparability of the mAb product throughout its development programme. Some changes in product characteristics can be anticipated – for example, following improvements in purification methods or conjugation chemistry. All such changes should be identified and presented in clinical trial submissions or during an application for a product licence and the implications of the change(s) should be discussed. It is not expected that process consistency will be demonstrated during early clinical development – partly because insufficient batches will have been produced to allow for adequate process validation, and because the process is likely to be subject to further optimization. However, the product must be demonstrated to be free from contaminants and sufficiently characterized to allow for bridging to subsequent clinical materials and the commercial product.

Expectations regarding how rigorously good manufacturing practices (GMP) will be implemented at the early stages vary among regulatory authorities, and consultation with the NRA early in product development is recommended. In some jurisdictions, process validation may be expected to address safety issues such as aseptic operations, sterile filtrations, cleaning validations, environmental control of facilities and validation of the process utilities (such as heating, ventilation and air conditioning (HVAC) systems, and water for injection or use in purified water systems).

During later clinical stages and at licensing submission, the manufacturing process may be firmly established and process-specific validation completed by demonstrating that a predefined number of consecutive full-scale commercial batches (usually at least three, unless otherwise justified) can be made that conform to predetermined criteria for batch processes. However, a continuous process-verification approach would be applicable to relevant manufacturing processes. Although a QbD approach is not considered in detail in the current Guidelines, such an approach is suggested during the development of manufacturing processes for mAb products (*38*) provided that the principles discussed throughout this document are adequately addressed.

Special considerations for analytical procedures and specifications

The testing of mAb substance(s) and of the final mAb product, as well as in-process control testing, may be expected to confirm the product safety of batches used in early clinical trials. In this regard, the NRA may expect that tests for bioburden/sterility, endotoxin and freedom from adventitious agents are developed, qualified and applied to each batch. Although other tests may not be fully validated, assay verification should have been performed even from an early clinical phase. This is likely to fall short of the full validation requirements detailed in ICH guideline Q2(R1) on the validation of analytical procedures (*39*) but should nevertheless give an indication that each method is fit for purpose.

Tests for safety, quantity, potency, identity and purity are essential requirements for any mAb product during its clinical development programme. Upper limits should be clearly established for the acceptable levels of both product- and process-related impurities, and be supported by published safety limits or data gathered during clinical trials. A justification should be provided for the quality attributes included in the specifications and for the acceptance criteria for purity, impurities (product- and process-related), quantity, potency and any other quality attributes that may be relevant to product performance. The justification should be based on relevant development data, the batches used in nonclinical and/or clinical studies, data from stability studies, and knowledge obtained during the production of similar molecules.

It is acknowledged that during early clinical development, the acceptance criteria may be wider than the final specifications for a product intended for Phase III studies and for commercial mAb production. During production of the batches intended for clinical trial use, not all attributes tested may have established specification ranges as the number of batches manufactured may be insufficient to determine the acceptable ranges. Nor at this time would a clinically meaningful range always be known. However, as the clinical programme continues – and prior to process performance qualification (PPQ) – specification ranges should be set for each attribute. Data from licensed mAb products made using the same platform technology and manufacturing process may be used as a guide to establish some specifications, such as the criteria for process-related impurities.

Product characteristics that are not completely defined in the early stages of development, or for which the available data are too limited to establish relevant acceptance criteria, should also be recorded. Such product characteristics could be included in the specification without predefined acceptance limits. At the initial stages of development, testing may not be required to determine residual levels of process contaminants (except residual host cell DNA (hcDNA) and host cell proteins (HCPs) if sufficient justification can be provided using a theoretical calculation. However, data demonstrating the adequate clearance of process-related impurities should be provided to support the licensing application.

For later-stage clinical trials, it is expected that all analytical procedures would be qualified for their intended purpose, and some NRAs may expect the methods to be validated. The specifications set for each parameter should be justified and capable of reflecting consistent process capability and product consistency in terms of quality, safety and efficacy. The specifications may be based on a number of factors, such as: (a) process and analytical method capabilities; (b) structure–function relationship studies; (c) manufacturing history; (d) historical release and stability batch data; (e) compendial requirements; and (f) clinical suitability and clinical experience. If justified, following the manufacture of additional batches of product, the sponsor should commit to revising the specifications as data on process capability and clinical outcomes (safety and efficacy) are accumulated.

During a public health emergency, data on clinical suitability are likely to be limited and should be considered to the extent that they are available. Under such circumstances, and with the appropriate rationale, data from related licensed mAb products manufactured using the same platform technology and manufacturing process may be considered during product development and evaluation. However, this strategy should be discussed as early as possible with the NRA.

Part A. Manufacturing and quality control

A.1 Definitions

A.1.1 International name and proper name

Historically, mAbs and mAb fragments have been assigned International Nonproprietary Names (INN) composed of a random prefix, infixes (which suggest target class and species or recombinant origin) and the stem "-mab" (*40*). With the increasing development of modified mAbs and mAb mimetic proteins (for example, conjugated mAbs, mAb fragments, multispecific mAbs, etc.) the naming structure for mAbs has evolved to reflect the growing diversity of related substances. In October 2021, the mAb nomenclature scheme of the WHO INN Programme was revised and the stem "-mab" discontinued and replaced with four new stems: "-tug", "-bart", "-mig" and "-ment" (*41*). The WHO INN Programme[20] regularly revisits and revises the naming scheme as required.

A.1.2 Descriptive definition

A mAb is, in general, a full-length immunoglobulin consisting of the Fc region and the antigen-binding domain comprising the Fab and Fv regions. Although the majority of commercially available mAbs are IgG isotypes, other isotypes are considered to be within the definition of a mAb. MAbs may be chimeric, humanized or fully human, and may also be genetically altered and/or chemically modified following their purification. MAb fragments consist of a section, or combination of sections, of the mAb (usually the Fab or Fv regions) or may be single-domain antibodies (variable domains of heavy or light chains).

MAbs are typically derived from the expansion in culture of a single clonal cell expressing an immunoglobulin with affinity to a unique epitope, or unique set of epitopes (for example, bispecific mAbs), and may be generated using a variety of methods, including hybridoma, phage display, humanized transgenic mouse technologies, single B-cell cloning or recombinant DNA technologies. MAbs may be produced in cultured mammalian cells (such as CHO, SP2/0 or NS0 cells) or human cell lines (such as PER-C6 or HEK), or in bacterial cells, yeast, fungi, plants or cultured plant cells. Preparations may be generated from cells or plants that produce only mAb fragments or genetically altered mAbs. Following purification, the mAb or mAb fragments can be further modified to alter their pharmacokinetic and/or pharmacodynamic profiles. Product formulations may also combine two or more mAbs and/or mAb fragments that each recognize different epitopes or antigens (referred to as co-formulated mAb products). Unless intended for non-parenteral administration, mAb preparations should be produced as sterile aqueous solutions or freeze-dried materials. Regardless

[20] See: https://www.who.int/teams/health-product-and-policy-standards/inn/

of their intended route of administration, all mAb products should meet the specifications set in accordance with the principles described in this section (Part A) of the current document.

Due to potential differences between similar mAbs or mAb fragments, a clear description and characterization of all active substance(s), and of the final product, must be provided to the NRA. This may include details such as structural characteristics, subunit details, antibody class/subclass, chemical modifications and conjugations, and amino acid sequence.

A.2 General manufacturing guidelines

The guidance provided in WHO good manufacturing practices for pharmaceutical products: main principles (*42*) and WHO good manufacturing practices for biological products (*43*) should be followed at establishments manufacturing mAb products intended for human use. These practices include the safe handling of all manufacturing reagents and organisms under appropriate containment conditions based on risk assessment and applicable national and local regulations (*42–44*).

Regardless of the manufacturing process, manufacturers must conduct a PPQ at commercial scale prior to marketing authorization. A predefined number of consecutive batches (typically three) are usually required, unless otherwise justified. All such batches should meet their specifications for both the substance(s) and product. The manufacturing process must be shown to consistently yield substance(s) and product of satisfactory quality as outlined in these Guidelines. All assay procedures used for the quality control of any intermediates, substances and final product should be validated at the time of commercialization.

The impact of post-approval changes to validated source materials, manufacturing processes, reference standards or quality control test methods should be assessed prior to implementation. If changes to the source materials or production process are made during the development programme or following marketing authorization, then pre- to post-change comparability studies of the substance and/or product must be conducted. These changes may require approval from the NRA prior to implementation (*45–47*). The number of batches used for a PPQ related to post-approval changes should be justified on risk-based and science-based principles. Although a minimum of three PPQ batches may be required for major quality changes, a lower number of batches may be acceptable for changes causing minimal expected changes to quality. Post-approval changes with a demonstrated absence of impact on quality may be monitored through continued process-verification only. Additional information on reporting categories and requirements for manufacturing changes can be found in the WHO Guidelines on procedures and data requirements for changes to approved biotherapeutic products (*45*).

For mAbs produced in plants, the application of GMP to upstream production processes (for example, plant cultivation, harvesting and initial

processing) may not be practical. Instead, a stringent quality system must be established and implemented prior to the marketing authorization of plant-derived mAbs. Although the WHO guidelines on good agricultural and collection practices (GACP) for medicinal plants (*48*) provides useful guidance in this respect, it is intended for use with medicinal plants (such as those used in traditional medicine) and is considered to be insufficient for establishing a stringent quality system for transgenic plant production systems.

The development, cultivation and use of transgenic (genetically modified) plants for the production of mAbs must conform to national and/or regional regulations and guidelines. When the bioengineered plant is from a species that is also used for food or feed, appropriate containment measures must be in place to ensure that there is no inadvertent mixing of the transgenic plant material with plants or plant material intended for food or feed use. Appropriate tests should be available that can detect the presence of the genetic insert and/or the product in the agricultural community. Proper environmental risk assessments must be conducted prior to growing transgenic plants in contained environments and their introduction to open fields. Additional resources and training on considerations in the use of transgenic plants can be found in the Biosafety resource book developed by the Food and Agriculture Organization of the United Nations (*49*).

A.3 Reference materials

Biological reference standards are used in qualifying or validating test procedures to ensure uniformity in the designation of potency or activity of biological preparations. These are required to ensure lot-to-lot consistency of production and to minimize the systematic deviation of assays. The WHO Recommendations for the preparation, characterization and establishment of international and other biological reference standards (*50*) describes the preparation of national standards.

A.3.1 International reference materials

WHO international reference standards and pharmacopoeial standards are available to support bioassays for some mAbs. These standards must be appropriately validated against clinical batch performance as part of the reference standard programme for the bioassay in order to avoid critical shifts in potency calculations. Relevant WHO international reference reagents may also be available from custodian laboratories for use in some quality control assays (for example, for antigens that may be used to coat binding plates for use in ELISAs). A catalogue of available WHO international reference standards is available on the WHO website.[21]

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

A.3.2 In-house and secondary reference materials

In-house and secondary reference materials should be established and maintained as described in the WHO Recommendations for the preparation, characterization and establishment of international and other biological reference standards (*50*) and as per the principles outlined in the WHO manual for the preparation of reference materials for use as secondary standards in antibody testing (*51*). For biosimilar products, the WHO Guidelines on evaluation of biosimilars (*52*) should also be consulted with regard to the appropriate selection and use of reference materials. If an international standard or reference material is not available, an aliquot of a product lot that met the specifications in place at the time of product release shall be used as an in-house reference material. The criteria for establishing manufacturer reference materials, and their specifications, should be approved by the NRA.

All reference materials must be assessed for their suitability for their intended purpose. Reference materials to be used in quantitative methods (for example, in the determination of potency) will require rigorous assessment to establish their true value. The number of determinations used to set the value must be statistically justified and take into consideration the inherent intra- and inter-assay variability of the method. The evaluation of all reference materials should include tests to assess their appearance, pH, protein concentration, identity, purity, and activity or potency. Biological reference materials should also be fully characterized, including any relevant structural characteristics and/or post-translational and chemical modifications. All reference materials must meet their specifications at the time of use.

The use of a two-tiered system consisting of a primary reference standard and a working reference material is strongly recommended. The primary reference standard should be used for the requalification of each working reference material batch, as well as to qualify future primary reference standards. Therefore, a full characterization of the primary reference standard is recommended that should take into account aspects such as higher-order structure, protein concentration, purity, quality and potency. Subsequent reference standards must be confirmed against the established specifications of the primary reference standard and their potency calibrated against that of the prior primary reference standard. The working reference materials should be evaluated using the same test methods as the primary standards, or by a subset of these methods, as well as any characterization methods relevant to its intended use. Further characterization may include post-translational modifications, thermal stability and isoelectric points. Where possible, it is recommended that the initial primary reference standard and working reference materials are established at the same time and from the same lot.

Reference materials should be requalified on a periodic basis determined from platform knowledge and/or risk assessment. If using a two-tiered system,

only the primary reference standard may need to be requalified. Data from the working material may be used if the primary and working standards had been produced from the same lot. For the requalification, quality attributes capable of assessing potential changes that may influence product quality should be selected. If the reference standard does not meet its specifications, it should be promptly replaced. The qualification programme should be prospectively put in place.

In cases where a mAb preparation has a short validity period (for example, radio-labelled mAbs), the reference material may comprise the unlabelled product and/or a product with a non-radioactive label/conjugate.

All reference materials should be stored under conditions that maintain their stability for use in assays of subsequent lots. Shelf-life and storage conditions for reference materials should be determined based on stability data.

A.4 Control of source materials

Consultation with the NRA is recommended regarding any novel expression system not discussed in the current document.

All materials used in the manufacture of the drug substance and drug product, as well as the point at which they are used in the manufacturing process, should be described in the submission for marketing authorization. This will include, but is not limited to, media components, enzymes, solvents, buffers, conjugation-reaction reagents and resins used in purification columns. Compendial grade raw materials should be used whenever possible and their grade provided. Information on raw materials of biological origin should include their source, grade (if relevant), control tests and specifications. Information on non-compendial grade raw materials used should include their control tests and specifications, and confirmation that they meet the standards appropriate for their intended use. For mAb conjugates, the quality control and characterization or testing of the linker molecule and the compound(s) to be conjugated to the mAb should also be considered prior to the conjugation process. The extent of the details required for materials used in the manufacturing process may vary between countries and should be confirmed with the NRA.

Manufacturers are encouraged to avoid the use of materials of animal origin. However, if the preparation of the culture medium, or downstream processing steps, involves materials of animal origin, these should comply with the *WHO guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products* (*53*). All materials of animal origin should be assessed for the risk of adventitious agents, which may include testing. The use of materials of animal origin should be discussed with and approved by the NRA. The culture medium used in the preparation of commercial product lots should also be free from substances likely to cause toxic or allergic reactions in humans. If culture media do include such substances, a risk-based approach, as

per ICH Q9 (*9*), should be followed, which may require applicable toxicological assessments and demonstration of clearance to below acceptable levels.

Manufacturers should take careful note that changing the mAb production cell line or cell type after their development will require the conducting of comparability studies between products derived from the previous and new expression systems. The comparability studies required would depend upon the developmental stage of the product, and should be risk based and commensurate with the magnitude of the change and its potential to impact product quality attributes. Although a combination of analytical testing and biological assays may be sufficient (*54*), it may also be necessary to conduct nonclinical and clinical bridging studies to demonstrate the safety, efficacy and bioequivalence of the mAb generated in the new production cell line system.

The specificity of the mAb should be verified during development of the cell or plant production system. Testing should include evaluation of the capacity of the mAb to react with the target antigen, and determination of its isotype and light-chain composition. Additional testing to verify mAb identity is also recommended and could include Western blot, verification of the mRNA by polymerase chain reaction (PCR), glycosylation analysis, and amino acid and/or peptide mapping analysis by mass spectrophotometry.

A.4.1 Generation of mAb expression systems using rDNA technology

MAbs manufactured using rDNA technology should be produced using reliable and continuous host cells or host plants. Details of the host cells or plants, including their origin, source and history, should be provided. All starting and source materials used in the growth and maintenance of the host cells should be adequately controlled.

Various prokaryotic and eukaryotic expression systems are available for use in the production of mAbs. Common prokaryotic cell lines include *Escherichia coli* (*E. coli*) and *Pseudomonas putida* and are often the system of choice for non-glycosylated mAbs and mAb fragments (*55*). Common eukaryotic systems include mammalian, yeast, fungal and insect cell lines, as well as plants. At the present time, CHO cells are the most commonly used expression system for the production of mAbs, although murine SP2/0, NS0 and human HEK293 cells also have a long history of use.

A.4.1.1 Expression vector and host cell

The process used for deriving the expression vector and selecting the host cell should be described in detail. The source and history of the host cell, as well as any prior genetic manipulation or engineering it may have undergone for its selection as a host should be included. Details should be provided on the vector, the identity of any cloned gene, and the genetic elements and functions of the

component parts of the vector. Important vector component elements to note include its origins of replication, any promoters and antibiotic markers, as well as a restriction enzyme map indicating the sites used in the development of the vector. The coding sequence for the expression vector should be understood and verification made that it is correctly incorporated into the host cell.

Details of the transformation into host cells, the rationale for the selection of the cell clone used for production, determination of whether the vector remains extra-chromosomal or integrated, and its copy number should all be reported. All measures used to promote and control the expression of the cloned gene should be described in detail.

A.4.1.2 Transgenic plants

The selected source plant should be capable of producing a consistent product when grown under its intended conditions in either a controlled environment or open field. Plants may produce secondary metabolites (for example, toxins or other bioactive substances) in response to their growing environment, stressors or genetic manipulation. It is therefore crucial to understand which relevant secondary metabolites the plant is capable of generating to ensure the implementation of proper downstream testing and purification processes.

Documentation should be provided which includes details on the characterization of the rDNA constructs or viral vectors, as well as any other genetic manipulations used to transfer genes into the plant. The stability of the gene expression system and its continuation through seeds or plant cuttings must also be clarified.

All materials used in the growth and maintenance of the plants (for example, fertilizers, substrates, pesticides etc.) should have quality attributes appropriate for the production of mAbs. Each lot of such material should be assessed for the presence of foreign matter. Care should be taken to minimize contaminants (for example, moulds and other agents) that could lead to the inadvertent exposure of product recipients to undesirable impurities, or that could affect product quality.

A.4.2 Generation of hybridomas for the production of mAbs

The methods used for lymphocyte isolation, fusion of lymphocytes with myeloma cells, immortalization of lymphocytes, selection of hybridomas and screening of mAbs must be recorded.

A.4.2.1 Material used for immunization

The antigenic material, including any adjuvant, used for the generation of immune lymphocytes should be defined. If the immunogen is derived from a human source, relevant clinical data on the donor(s) should be recorded.

A.4.2.2 Immune parental cells

Where possible, the source of the immune parental cells should be documented. For murine mAbs, information on the animal strain should be provided, including its specific-pathogen-free (SPF) status. Where possible, animals used for immunization should be SPF.

For human immune parental cells, all data relevant to possible viral infections of the human donors should be available. The donated samples of immune parental cells should be screened for potential viral contamination, and in accordance with national requirements for blood donations and the use of blood products.

A.4.2.3 Immortalization procedures

For animal cells and animal-derived cell banks, reference should be made to the WHO Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (*56*). Where myeloma cells are used, they should be fully described, including details of their source, origin, history, name and characteristics, as well as the storage culture conditions used in their expansion prior to fusion. It is preferable to use immortalizing cells that do not synthesize immunoglobulins themselves.

Human B lymphocytes are usually immortalized by infecting them with Epstein-Barr virus (EBV) – however, this procedure alone cannot always ensure stability, and subsequent fusion with a myeloma may be required. If EBV is used for immortalizing human B lymphocytes, its origin and characteristics should be clearly specified.

Before being fused or immortalized, cell cultures should be tested for sterility according to the WHO General requirements for the sterility of biological substances (*57*, *58*) or using suitable methods approved by the NRA. All cells should be found to be negative for bacterial, fungal, viral and mycoplasma contamination. Any identified viral contamination (for example, EBV) should be documented and the risk assessed to ensure its control and demonstration of removal during downstream processing.

A.4.3 Animals used for mAb production

The use of animals in the production of mAbs for use in humans is strongly discouraged. If animals are intended to be used for the production of mAbs from their ascites, they must be from SPF-monitored colonies and free from viruses for which there is evidence of capacity to infect humans or primates. Both the animals and the cells injected into the animals should be tested for appropriate viruses using PCR or other nucleic acid amplification methods. If animals are

found to be infected with viruses for which there is no evidence of capacity to infect humans or primates, the final product may be accepted only if the purification process has been demonstrated to eliminate the infecting virus(es).

A.4.4 Cell or seed bank system

The production of mAbs should be based on a cell or seed bank system consisting of a master bank and working banks. Cell cultures or plants derived from the working bank should have the same characteristics as the cell cultures or plants from which the master bank was derived. Information on the establishment, characterization and cloning of the original cell or seed line used to establish the bank shall be provided. As described in the WHO Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (*56*), a single cell clone should be isolated for expansion into a cell bank regardless of the source of the cells.

The use of a stable cell pool in lieu of a clonally derived cell bank may be considered for early clinical batches to reduce product development time. The use of high-density CHO cell cultures and transient expression processes may also be considered to speed up production and evaluation. However, the use of either of these strategies should be discussed with the NRA.

Once a pure culture is established, it should be sub-cultured for production into a master cell bank (MCB). The use of an MCB system will reduce the risk of future contamination or loss of the pure culture. It is strongly recommended that a two-tiered cell bank system is used with working cell banks (WCBs) being derived from the MCB. Although the use of a one-tiered system containing only an MCB is acceptable, this must be justified.

During product development, the production system should be demonstrated to yield a mAb of consistent quality. The cells at the limit of in vitro production (that is, end-of-production cells) should be characterized to demonstrate such consistency as per ICH guidelines Q5B (*59*) and Q5D (*60*). Consistency of the coding sequence of the expression construct should be verified in cells cultivated to the limit of their in vitro cell age for production use or beyond.

For transgenic plants, the stability of the transformant should be established. Once verified, a two-tiered master seed bank (MSB) and working seed bank (WSB) system should be employed.

Details of the cell or seed bank system should be well documented and include information pertaining to intended cell bank use, size, types of containers and closure systems, development of the cell bank(s), cryoprotectants, media used, culture or growth conditions, long-term storage conditions and evidence of stability of the expression system under those conditions. Long-term

stability monitoring plans for cell or seed banks should also be established and documented in marketing authorization applications. It is expected that all cell and seed banks will be monitored for their viability and ability to produce the desired product in order to demonstrate and ensure their stability.

A.4.4.1 Control of master cell or seed banks

All cell banks, regardless of cell type, should be tested early in the development programme to confirm their identity, purity and presence of the appropriate product-specific coding sequence, as well as to establish the suitability of the cell system for the production of mAbs of consistent quality. The extent of cell characterization during the development process can influence the type and degree of routine testing needed at later stages of manufacturing. Additional recommendations on tests for the characterization of cell banks is provided in Part B of the WHO Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (*56*).

Cell banks should be free of any detectable adventitious agents in accordance with ICH Q5A (*61*). Bacterial cell banks must also be free from bacteriophages. For MSBs, the level of bioburden should be controlled.

A.4.4.1.1 *Identity tests for substrates*

The identity of all cell and seed banks must be confirmed. The selection of appropriate identity test methods will be influenced by the cell or seed type, culture or growth conditions, available resources, and whether other cell cultures or plants are maintained in the same facility. Acceptable methods include:

- phenotyping
- isoenzyme analysis
- karyotyping
- human leukocyte antigen typing
- gene sequencing or next-generation sequencing
- short-tandem repeat analysis.

Phenotyping methods (such as the observation of cell or plant morphology and growth curve analysis) can provide early feedback on the performance of a cell or seed bank and can help identify problems that may arise in their stability during storage. Isoenzyme analysis can identify the species of origin but does not distinguish cross-contamination with other cell lines from the same species. The selection of appropriate genetic analysis will largely depend on the type of cells or plants used in the manufacture of mAbs, the risk of cross-contamination

with other cells, and the risk of genetic changes occurring during storage or growth. Gene sequencing or next-generation sequencing can range from a single gene analysis to whole-genome sequencing, with the latter being recommended for microbial cultures (using verification against published reference genome sequences).

A.4.4.1.2 *Tests for microbial contaminants*

All cell banks should be tested for relevant bacterial, fungal and/or viral contaminants. An assessment of specific viruses (and families of viruses) that may potentially contaminate the cell bank should be conducted to help guide the selection of appropriate panels for testing. Mycoplasma contamination should be tested for in MCBs. Appropriate tests for detecting mycoplasmas include direct and indirect culture methods and PCR assays. Bacterial cell banks should be tested for bacteriophages and a bacteriophage contamination protocol should be in place in the event of such contamination being detected.

For plant seed banks, the level of bioburden should be controlled.

A.4.5 Control of working cell or seed banks

It is expected that during the product life-cycle the WCB/WSB will become exhausted and require replacing. Control of the WCB/WSB should be in accordance with a protocol agreed upon with the NRA. All new WCBs/WSBs should be appropriately characterized and qualified prior to use as per ICH guidelines Q5B (*59*) and Q5D (*60*), including through tests of purity and identity (*56*). Additional information on reporting categories and requirements for the generation of new WCBs/WSBs can be found in the WHO Guidelines on procedures and data requirements for changes to approved biotherapeutic products (*45*).

A.5 Control of mAb substance production

The manufacturing process must be validated before licensing, including through an evaluation of all process steps to ensure they consistently yield a substance and product of acceptable quality. During the development programme, it will be important to establish an understanding of the heterogeneity of the mAb(s), and of the impact of process changes on the heterogeneity profile of the substance. As the field of analytical chemistry and technologies is advancing rapidly, only some of the more commonly used methods for the analysis of mAb structure, function and quality are mentioned in this document. The implementation of new or novel analytical technologies should be discussed with the NRA.

While manufacturing details and safety issues may differ between the various expression systems, some general principles can be applied. Appropriate

in-process testing should be selected which takes into consideration the potential safety concerns of the expression system used.

Steps should be taken during manufacture to control bioburden and prevent contamination with viruses, bacteria, fungi, mycoplasmas and the prion responsible for TSEs.

Production techniques should be used that have been demonstrated to minimize any impurities inherent in the production processes, and that prevent the introduction of contaminants external to the manufacturing process. Appropriate risk assessments for impurities should be conducted during process development and/or validation. Potential impurities and contaminants which should be considered include:

- peptides and proteins which do not constitute the substance or drug product;
- host cell proteins (HCPs);
- residual host cell DNA (hcDNA);
- endotoxins and non-endotoxin pyrogens;
- culture media constituents and additives;
- components that may be extracted or leached from contact surfaces during production and purification steps (for example, reactor vessels, transfer tubing, purification columns and storage containers); and
- reagents from any conjugation process, as well as substances which remain unconjugated.

Emphasis should be placed on minimizing the risk of contamination from the environment or cross-contamination with other products. The operational and design features of the purification suite, HVAC and other support systems, equipment, transfers of any intermediates or substances, and movement of personnel should also be considered.

A.5.1 Production of mAb substance

A.5.1.1 Production from cell cultures

Only cultures derived from a qualified cell bank shall be used for production. The use of chemically defined and serum-free growth media is preferred over media containing animal serum. If animal serum is included in the medium used for the production of cell cultures, it must be tested to show its freedom from bacteria, yeast, fungi, viruses and mycoplasmas. Each batch of serum shall be of certified origin and, if bovine, shall come from herds certified by the appropriate authority to be free from TSE (*53*). Test results provided by the supplier of the

serum may be sufficient if the tests were performed according to validated and well-documented procedures. Similar control measures and testing procedures should be implemented for any animal-derived substance (for example, porcine trypsin) which may be used in the production of mAbs.

Appropriate in-process controls and monitoring programmes should be in place to ensure the production of consistent substance(s). The consistency of the growth of the production strains should be demonstrated by monitoring their growth rate, pH, pO2 and the final yield of mAb substance – however, monitoring should not be limited to these parameters and should be conducted based on consideration of the cells and cell culture system used.

Samples from the culture system should be taken during and/or at the end of production and examined for viruses and other potential adventitious agents as per ICH Q5A (*61*). If the cells are to be inactivated or lysed prior to purification, the samples should be taken before such steps. The purity of the culture should be verified using suitable methods, such as inoculation into appropriate culture media. If contamination is found, the culture and any substance or product derived from it should be discarded.

If an inactivation or cell lysis method is used, it should be monitored to ensure completeness using a validated test during routine manufacturing. If a chemical agent is used for cell inactivation or lysis, validated methods for its detection must be in place and residual levels should be controlled. The impact of the inactivation process on mAb heterogeneity should also be evaluated.

A.5.1.2 Production in transgenic plants

For each process that is not intended to be sterile, extraneous bioburden should be controlled using procedures to minimize the introduction of potential contaminants, and through in-process testing.

For greenhouse-grown material, the types of containers, soil mix composition and greenhouse growth conditions can impact product quality. For field-grown material, the previous uses of the land (for example, agricultural and/or industrial use) can also affect product quality and should be documented. Specifications, acceptance criteria and other limits should be established for the soil composition and potential soil contaminants that may affect production. In addition, controls on the agricultural methods used during crop growth, including specifications regarding the use of chemicals and limits on specific agricultural practices (for example, the use of specified fertilizers, pesticides, or herbicides, and irrigation practices relative to a specified harvest time frame, etc.), should be in place. All pest-control measures implemented should be in accordance with national and/or regional agricultural requirements and best practices.

For field-grown plants, control must be maintained over the growing process from planting through to harvesting, and over the disposal of remaining

crops and/or crop residue and, if required, over the subsequent use of the field if used for growth of food or feed, or as a pasture, during subsequent seasons. Control measures should include the accounting of seed that is transferred from seed bank storage to the field for planting, or for archiving. Records should be maintained on plant growth rates, environmental conditions (for example, daily mean temperatures, rainfall and sunlight hours), and the presence of weeds and insects (or other animals) from the time of planting to harvest. Conditions for determining when the plants are to be harvested should be clarified prior to planting.

Documentation on the size and location of all sites where the bioengineered plants will be grown, on the control of pollen spread, and on the subsequent use of the field and destruction of volunteer plants in subsequent growing seasons should be maintained and provided to the NRA. Such documentation may also be required and/or requested by other national regulatory agencies such as those for the environment, wildlife or agriculture.

Appropriate containment procedures should be in place for the transport of the source material from the field or greenhouse to the production facility. During transport, containers of harvested material should carry a label that clearly indicates that the material is not to be used for food or feed purposes.

In-process wastes, rejected in-process material and residual source plant material from the purification process should be treated to inactivate the regulated product prior to its disposal. The waste should be disposed of in a manner that ensures that the material will not enter the human or animal food chain, and in accordance with national and local regulations and best waste-management practices.

In-process monitoring of sterility or for mycoplasma contamination would be inappropriate for any green plant material prior to appropriate purification and filtration steps. However, appropriate measures should be in place to minimize bioburden or other extraneous contamination.

A.5.1.3 Production from ascites

The production of mAbs in animal ascites for use in humans is strongly discouraged – however, it is recognized that this method may be required under unique circumstances but in such cases strong scientific justification should be provided for not using in vitro cell culture or plant-based methods. When the ascites method of production is used, the 3Rs principles ("Replace, Reduce, Refine") should always be followed to minimize the use of animals. Discomfort, distress and pain must also be avoided as much as possible, and any animals under distress should be euthanized.

Animals should be weighed prior to injection of hybridomas and their weight gain monitored daily. If substances other than pristane are used to prime the animals to facilitate the growth of hybridomas, the NRA should first approve

them. Harvesting of the ascites fluid should be done under anaesthetic and before the abdominal distension becomes distressful to the animal and before its normal activity, respiration, and food or water intake are negatively impacted. A maximum of four harvests (taps) may be drawn from the same animal prior to its euthanization. If the collected exudate is bloody or cloudy it must be discarded and the animal humanely euthanized immediately.

A.5.2 Conjugation

Several multi-step chemical and enzymatic methods for the conjugation of mAbs to small-molecule drugs (*62–65*) or PEG (*66–68*) have been described. The choice of conjugation process should be justified taking into consideration the purpose and function of the final product. The linker selected should remain stable during circulation in the blood so as not to inadvertently release the conjugated payload prior to reaching the target of interest.

Methods that conjugate in random positions on the mAb are less desirable due to the generation of broadly heterogeneic conjugate mixtures with variable lot-to-lot consistency, pharmacological effects, potency, efficacy and stability. Methods employing more-specific conjugation chemistry allow for better control of the site of modification and ratio of payload to mAb, and result in better batch consistency. The method selected for conjugation should be approved by the NRA.

All individual components used in the conjugation process must be controlled for identity, purity and stability. The potency of the mAb and the content of a pharmacologically active payload should also be confirmed prior to their conjugation. PEG molecular size distribution, monofunctionality, and linearity or branch size should be verified. Characteristics that contribute to the safety, efficacy and stability of the mAb conjugate should be determined during the development process as these will be important in substance and product control. Such characteristics can include the ratio of payload to mAb, potential conjugation sites, unintended or incomplete conjugations, impact of conjugation on mAb recognition of the antigen-binding site and its affinity, functionality of the Fc region, and changes to size or charge variants. Due to the increased complexity of conjugated mAbs, multiple assays are likely to be needed to ensure that all aspects of their mechanism of action are properly controlled.

Both the conjugation method and the control procedures should be well established to ensure the reproducibility of the reaction and the production of safe and stable mAb conjugates prior to their clinical evaluation. The same conjugation process should be maintained throughout the development programme and commercialization. The method should be monitored and analysed for any unique reaction by-products (as well as residual un-reacted functional groups or their derivatives) that are potentially capable of reacting in vivo and may be present following the conjugation process. The manufacturing

process should be validated and the limits for reaction by-products, as well as un-reacted activated functional groups remaining at the end of the conjugation and purification processes, should be agreed with the NRA. For radio-labelled conjugates, the development and validation of the conjugation process may be performed using equivalent non-radioactive labels.

A.5.3 Purification

The purification process must be demonstrated through specific validation studies to be able to consistently remove, or reduce to an acceptable level, all product- and process-related impurities. The types of impurities that should be considered will largely depend on the materials used in production (for example, cell type, growth media, additives, etc.). The types of impurities to be considered and their acceptable levels should be based on an appropriate risk assessment. The implementation of sensitive detection and quantification methods will be crucial for the successful validation of the purification process and for understanding its capacities and limitations.

The purification of mAbs is usually performed over multiple steps using a combination of methods that may include centrifugation, filtration, ultrafiltration, affinity chromatography (for example, protein A or protein G), and ion exchange chromatography, or other liquid chromatographic methods. The conditions for each purification step should be clearly defined and should be based on knowledge generated during the development of the product and process. The use of existing platform data for purification process validation (for example, on impurity clearance) obtained for other mAb products manufactured using the same processes can be considered if justified and may help reduce the duration of product development. However, this strategy should be discussed with the NRA as early as possible during the product development process.

A.5.3.1 Product-related impurities

The purification process should be assessed for its capability to consistently reduce to acceptable levels the product-related impurities (for example, undesired heterogeneic mAbs, aggregates and fragments) which may occur during the production process.

Some conditions of purification have been documented that induce the formation of product-related impurities. For example, the aggregation of mAbs can be induced by some environmental and/or chemical conditions during chromatography or virus-inactivation steps. Therefore, the impact of each purification step on mAb quality must be evaluated. An understanding of the mAb chemistry and nature of the impurity can help in elucidating the mechanism of its formation during purification, optimizing test methods to monitor for its occurrence, and providing a means for mitigating the issue.

A.5.3.2 Host cell proteins

Host cell proteins (HCPs) will likely constitute the largest percentage and most physicochemically diverse range of the impurities that must be removed during production of the drug substance, and their diversity will vary according to whether cell or plant types are used, growth conditions, whether the mAb is secreted or derived from lysed cells, and any pre-purification processing steps. The control of HCPs is crucial in order to avoid their potential for inducing an undesired immunological response, prevent any adjuvant effect they may confer on the mAb substance, and prevent their potential impact on mAb substance quality (for example, through degradation by enzymatically active HCPs).

Commercially available ELISA kits for HCPs may be used for their quantification – however, they may not detect a sufficient range of proteins and should be carefully assessed for their capacity. Product-specific HCP antiserum may also be developed and qualified for use in an HCP ELISA prior to seeking marketing authorization. Additional discussion on HCP detection methods is provided below in section A.5.6.8.1.

A.5.3.3 Residual host cell DNA

Acceptable limits on the amount of residual host cell DNA (hcDNA), as well as points to be considered concerning the size of residual hcDNA in an rDNA-derived biotherapeutic, are discussed in section 5.2.2 of the WHO Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (*56*). These Recommendations indicate an acceptable upper limit of residual hcDNA of 10 ng per parenteral dose – however, it is important to take into consideration additional factors such as DNA fragment size, as well as any inactivating steps that may be included in the manufacturing process. Acceptable daily and/or treatment cycle limits for residual hcDNA should be discussed with the NRA.

A.5.3.4 Viruses

Purification processes should include dedicated manufacturing steps that are capable of removing or inactivating viruses when such a risk exists. Viral clearance studies must be conducted on the ability of the manufacturing process to remove and/or inactivate viruses and viral particles as described in ICH Q5A (*61*). The viruses used in clearance studies should resemble viruses known to, or likely to, contaminate the production cells, source material or other products which may be used in the production process. The viruses selected for viral clearance studies should be clearly justified.

Platform data already obtained for viral clearance validation for other mAbs can be considered when scientifically justified, and should be discussed with

the NRA. A generic viral clearance study may be considered where virus removal and/or inactivation are demonstrated for a model mAb in the purification process. The data may then be extrapolated to other mAbs manufactured using the same purification processes and virus removal/inactivation schemes as the model mAb.

If a viral genome is found in the MCB, the acceptability of the cell line for use in the production of the mAb should be discussed with the NRA. Additional guidance can be found in ICH Q5A (*61*).

A.5.3.5 Other impurities

Validation studies must also be conducted to ensure the consistent removal to acceptable and safe levels of other relevant impurities that may arise during the production process. These can include, but are not limited to:

- additives that may be present in the culture media or bioreactors (for example, antibiotics, insulin, IPTG, dimethyl sulfoxide, antifoam agents, serum-derived antibodies and serum substitutes);
- enzymes which may be used for digestion purposes;
- agents used in the purification process and columns (for example, protein A, and solvents used in running or elution buffers);
- reagents used in conjugation reactions, including unconjugated linker, drug and/or PEG components; and
- extractables and leachables derived from surfaces contacted during production and purification.

For mAbs produced in transgenic plants known to generate toxins (for example, protease inhibitors, haemolytic agents and neurotoxins), analytical testing, animal testing or validation of removal may be required to establish that any residual toxin levels are within a safe range in the final product. Where fertilizers, pesticides and/or herbicides may have been used on the plants or production fields, validation of their removal during the purification process may be an acceptable alternative to any corresponding final product safety tests. This should be discussed with the NRA. Plants may also produce proteases or other enzymes that can cause degradation of the drug substance and/or impact long-term product stability and so care should be taken to eliminate these as early in the purification process as possible.

A.5.3.6 In-process hold time

In-process substances may be held under appropriate conditions prior to further purification and/or processing steps – however, all selected hold times and their conditions must be validated, and must be approved by the NRA. Hold times and conditions should be supported by data which reflect all stability-indicating

attributes and which may be derived from small-scale studies, clinical trial manufacturing experience and/or commercial production. Non-sterile in-process substances must not be held under conditions that would support the growth of potential microbial contaminants. It is recommended that hold times are validated for microbial control at production scale with the exception of low-risk hold conditions such as: (a) intermediates which are non-growth promoting; (b) hold times which are below 24 hours; and (c) intermediates that can be held in sterile single-use bags after bioburden removal filtration. The methods used to control and monitor the bioburden of non-sterile in-process substances must be clearly defined.

A.5.4 Intermediates

If the mAb is intended to be modified after purification (for example, by conjugation), it is considered to be an intermediate prior to such modification. In general, the intermediate should be controlled as per the purified mAb substance – however, some testing may be reduced or delayed until after conjugation or other modification.

A.5.5 Drug substance filling and storage

The relevant guidance provided in WHO good manufacturing practices for pharmaceutical products: main principles (*42*) and WHO good manufacturing practices for biological products (*43*) should be followed with regard to the filling and storage of the drug substance.

All containers and container closure systems must be tested for their compatibility with the drug substance(s) and must also be in compliance with the requirements of the NRA (such as those for biological reactivity, leachables and extractables). Assurance of the absence of the prion responsible for TSE (*53*) should be provided if any animal-derived materials (for example, colourants made from tallow, or fatty acids used in polymer production) are used in the manufacture of the container or closure. The container should prevent microbial contamination of the mAb substance during storage.

The storage environment and conditions should not adversely affect the quality of the mAb substance. As mAbs in high concentrations have a propensity to form aggregates, particular care should be taken during any freezing process in which aggregation is more likely to occur. The manufacturer should provide the NRA with data that support the stability of the substance under appropriate conditions of storage and, if relevant, during its shipping.

A.5.6 Control of mAb or mAb conjugate drug substance

Extensive characterization studies should be conducted on the mAb or mAb conjugate drug substance during the development process, with the aim of

identifying critical quality attributes. Similarly, process-development studies should be conducted to identify individual steps that may impact upon the drug substance and thus upon product quality and stability. Appropriate in-process control strategies should be established during product development and updated, as needed, through its life-cycle. Control strategies should be based on knowledge gained through the characterization studies, experience in the manufacturing process and risk assessment, and must be scientifically justified. Additional information on the control of the drug substance may be found in relevant ICH quality guidelines (*8*, *9*, *11*, *69*).

At a minimum, characterization of the purified mAb drug substance should include physicochemical analysis, biological activity, purity, impurities, contaminants and quantification. A detailed discussion on characterization is provided in Appendix 2 of the WHO Guidelines on the quality, safety and efficacy of biotherapeutic protein products prepared by recombinant DNA technology (*2*). For mAb conjugate drug substances it is important to also understand the chemistry and control of the conjugation process in order to achieve a consistent conjugated substance, and to understand the impact of conjugation on the functions of the mAb and payload.

The appropriate testing requirements and specifications for the control of purified mAb and mAb conjugate drug substances should be determined during the characterization process and should reflect the identified critical quality attributes, production and purification processes, and any chemical or enzymatic modifications and/or conjugation reactions. Further discussion on such specifications is provided in Appendix 3 of the WHO Guidelines on the quality, safety and efficacy of biotherapeutic protein products prepared by recombinant DNA technology (*2*). All methods intended for quality control purposes must be demonstrated during the development process to be suitable for their intended purpose, and be validated prior to application for marketing authorization. The control strategy (test methods and specifications) for the mAb or mAb conjugate substance should be discussed with the NRA. Purified mAb and mAb conjugate substances should be evaluated for the following attributes at release – however, where justified, some tests may be conducted at an appropriate manufacturing step. Once consistency of production has been demonstrated, it may be possible to omit some tests if sufficient justification is provided.

A.5.6.1 Appearance

The appearance of the purified mAb or mAb conjugate should be examined using a suitable method and should meet the established specifications for its physical state (for example, solid or liquid) and colour. For a dried or lyophilized preparation, the appearance should also be examined after reconstitution with the appropriate diluent and should meet the established specifications.

A.5.6.2 Identity

The identity test(s) selected should be specific and based on one or more properties of the mAb or mAb conjugate – such as its immunological specificity, molecular structure, isotype, light chain composition and/or presence of any conjugated payload. More than one identity test may be necessary, with the selected tests exhibiting sufficient specificity to distinguish between the mAb and other products that may be manufactured in the same facility, and between conjugated and non-conjugated mAbs.

A.5.6.3 pH

The pH of each batch should be tested. The results obtained should be within the established range based on the formulation pH target as supported by formulation development data and stability studies.

A.5.6.4 Protein concentration

Total protein concentration should be measured using a validated method of suitable sensitivity and specificity, such as determination of absorbance at 280 nm based on the protein-specific absorbance.

A.5.6.5 Potency

Potency assays should provide a quantitative measure of the activity (or activities) of the mAb or mAb conjugate relevant to its mechanism of action. The use of assays that reflect the mechanism of action in the clinical situation is preferable but not always possible or necessary when the assays are intended for quality control and release testing purposes. Multiple potency assays may be required to assess all of the relevant functions of the drug substance. This could include, for example, assays for binding to the target antigen as well as evaluating Fc function. For bispecific or multispecific mAbs, the capacity for dual or multiple binding to each of the target antigens would need to be confirmed.

Potency assays should be sufficiently sensitive to detect any differences in the mAb or mAb conjugate of potential clinical importance. Potency assays are also an important measure of manufacturing consistency and should be sensitive enough to detect changes in the mAb or mAb conjugate that may impact its activity and function(s), such as binding affinity or ADCC. The more direct assays for assigning potency to mAbs or mAb conjugates are usually in an ELISA format to assess binding capacity to the relevant antigen(s). Potency assays may be technically complex and involve, for example, SPR or flow cytometry, but may also be cell based – for example, by using a reporter cell line or measuring virus neutralization. Potency assays that include effector functions (such as CDC, ADCC or ADCP mechanisms of action) should also be considered where a mAb

activity is dependent on more than antigen recognition and binding. The selection of appropriate assay(s) for monitoring the potency of the drug substance should be discussed with the NRA.

For cell-based assays that use a continuous reporter cell line, a cell bank system should be generated and qualified. Any reporter gene function should also be shown to be stable during cell storage and growth. For virus neutralization assays, an MCB of virus producer cells should be appropriately qualified and used to generate a WCB.

For mAb conjugates in which the payload has specific pharmaceutical properties (for example, drug or radio-labelled conjugates) the assignment of potency should take into consideration the binding and any effector function of the mAb following conjugation, the pharmacological activity of the payload and the payload-to-mAb ratio. For some mAb conjugates, the use of a cell-based assay for assigning potency may be sufficient – for example, when the mechanism of action reflects both the mAb binding and payload functions.

Potency assays for mAbs are usually expressed as a percentage of activity relative to established and qualified reference standards which themselves are linked to product batches used during preclinical and clinical trials. Specifications should take into consideration historical release and stability batch data, clinical experience, manufacturing history and capability, as well as the analytical capability of the methods used. Acceptance criteria that are outside the specification range should be justified based on the assay type, assay variability and historical batch data of the mAb.

Although in vivo methods can be used to determine potency, such assays are mostly done during the product development phase and may be inadequate for the purposes of quality control and release testing. Animal-based potency assays tend to have a much higher variability and may lack the sensitivity necessary for assuring the consistent quality of a mAb. If in vivo methods are used, it is important to ensure that the mAb target(s) are expressed within the animal and that any inter-species differences between the animal and human biology are considered. The selection and use of animal methods for release testing purposes must adhere to the 3Rs principles ("Replace, Reduce, Refine") to minimize the use of animals in testing, and should also be discussed with the NRA.

A.5.6.6 Heterogeneity profile

Following purification, purified mAbs and mAb conjugates will comprise heterogenous populations of molecular species with variants in mass, charge, glycosylation and other parameters (*19*, *26*, *70*, *71*). The types of variants encountered can be influenced by a number of factors, including the cell or plant substrate, culture media and environmental conditions during growth, and the purification processes, as well as by any additional chemical or enzymatic

modifications. Collectively, the variants provide a heterogeneity profile or "fingerprint" that is unique to each substance and manufacturing process, and on which the substance specifications are based. Heterogeneic variants that have biological activity comparable to the intended mAb product are considered to be product-related substances (*69*).

At a minimum, and as appropriate and justified, the heterogeneity profiles of each batch of purified mAb or mAb conjugate substance should be assessed. This could include, for example, assessment of mass (monomer purity), charge and glycosylation variants. An evaluation of the distribution of conjugate variants should also be conducted for purified mAb conjugates. Although a number of techniques may be used to measure the heterogeneity profile, the more commonly used quantitative tools include various high-performance liquid chromatography (HPLC) techniques (such as anion exchange, cation exchange, size-exclusion, and reversed-phase chromatography), capillary electrophoresis and isoelectric focusing electrophoresis (*71–74*). Nuclear magnetic resonance, mass spectrometry and circular dichroism may provide additional characteristics and information regarding the types of heterogeneic mAbs. A variety of analytical methods should be used to assess the heterogeneity profile and to establish acceptable limits. The selection of appropriate methods for evaluating mAb heterogeneity and acceptance criteria should take into consideration the mAb mechanism of action, quality, safety and efficacy, and may need to be discussed with the NRA.

If the purified mAb or mAb conjugate substance is well characterized and the manufacturing process has been demonstrated to be well controlled, then it may be feasible to reduce the number of tests required for purity and heterogeneity assessment. However, any subsequent changes to the materials used, equipment, manufacturing process, purification method and/or conjugation chemistry may warrant the re-establishment of appropriate tests.

A.5.6.7 Product-related impurities

Product-related impurities should be monitored for each batch of purified mAb or mAb conjugate. The impurities to be monitored should be consistent with those identified as critical quality attributes. Such impurities may include fragmented substance, aggregates, charge variants, chemical variants, post-translational modifications and/or glycoforms. For purified mAb conjugates, any unconjugated mAb, free payload and/or free linker–payload conjugate would also be considered to be product-related impurities. Appropriate specifications should be established and be based on knowledge gained during characterization studies, stability batch data, clinical experience, manufacturing history and capability, analytical method capability, regulatory expectations, safety and any compendial requirements for protein-based products.

The methods employed for assessing the heterogeneity profile (see section A.5.6.6 above) may also be useful for monitoring product-related impurities.

A.5.6.8 Process-related impurities

The selection of appropriate tests for the detection of process-related impurities should take into consideration all manufacturing steps starting from the WCB/WSB. The methods chosen must demonstrate sufficient sensitivity to detect levels which may be clinically meaningful. Acceptable limits for such impurities should be based on compendial requirements and clinical experience (justified by the minimum levels achievable by the purification process), as well as on their concentration after dilution of the purified mAb or mAb conjugate into the final product, the volume of administration and whether the product is intended for single or repeated administration. Testing for process-related impurities may be omitted when the levels achieved are consistently demonstrated to be significantly lower than the acceptable limit. Specifications for the impurities should be discussed with the NRA with acceptable limits based on the risk assessment.

A.5.6.8.1 *Host cell proteins*

HCPs are most commonly measured using an ELISA platform with polyclonal anti-HCP antiserum. As the performance of the assay is limited by the quality and specificity of the antiserum, the results will not accurately reflect the true level of HCPs if the antiserum does not recognize the majority of the HCPs or if the signal is dominated by antibodies to only a few of the proteins present in the sample being tested. Although commercial assay kits are available for the detection of HCPs from some cells (for example, CHO cells and *E. coli*), these assays may not detect HCPs unique to the cells grown under a manufacturer's bioreactor environmental conditions, or be sufficiently specific for the HCPs that may co-elute with the mAb during purification. The development of process-specific HCP antiserum raised against a harvest and/or from early in the purification process and from the same production cell line as that used in the manufacture of the mAb but transfected with an empty vector is recommended. HCP antiserum generated for one mAb product may be suitable for use across common technology platforms that utilize the same cell line.

The characterization and determination of the estimated percentage coverage of the HCPs detected by the antiserum/ELISA are important and should be provided during the application for marketing authorization. The use of two-dimensional gel electrophoresis methods is useful in this exercise but does not in itself provide sufficient information. Mass spectrophotometry analysis of HCPs is recommended as an orthogonal approach in order to identify individual proteins, quantify the more abundant ones and thus support the risk assessments.

Although there is no clearly established safe or acceptable level of HCPs, achieving levels below 100 ppm (< 100 ng/mg mAb protein) is generally recognized as sufficient. However, the acceptable level for any mAb product must be based on a risk assessment and will also depend on dose and frequency of administration.

A.5.6.8.2 *Other process-related impurities*

Other potential process-related impurities from the cell culture to consider include residual hcDNA, cellular metabolites and cell culture media components. A nucleic acid amplification technique (such as qPCR) or some colorimetric methods may be suitable for detecting and quantifying the level of any residual hcDNA in the purified mAb or mAb conjugate. Testing for beta-glucans should also be considered, particularly if the host cell is known to generate the oligosaccharide or if cellulose filters are used downstream (*75*).

Validated quantitative methods must be in place to test for trace levels of any antibiotic used in the culture media or at any other step in the manufacturing process. Similarly, methods should be established for the detection and quantification of cell culture components such as inducers, enhancing agents, surfactants, antifoam reagents, chelators and solvents. As the purified mAb or mAb conjugate substance can be a difficult matrix for some detection methods, the sensitivity of the technique should be demonstrated through spike-and-recovery studies.

Downstream processing steps, such as purification and conjugation, are likely to be important sources of impurities such as enzymes, chemical or biochemical processing reagents, buffer components, stabilizers, leachates, elemental impurities, chromatography media (such as organic solvents or dimethyl sulfoxide) and ligands which may leach from affinity columns (for example, protein A or protein G). For conjugated mAbs, methods must be in place to detect residual unbound payload, as well as the linker arm and all reagents used in the conjugating reaction.

For plant-derived mAbs, methods should be established and validated for the detection of any fertilizers, herbicides and pesticides which may have been applied to the field or to the crop before or during the growth of transgenic plants used in the production process. The potential for plants to produce agents which may pose a safety risk to humans or impact upon product quality or stability should also be assessed and identified during the development programme. Such plant-derived agents can include proteolytic enzymes, lectins, polysaccharides and/or secondary metabolites.

A.5.6.9 Sterility or bioburden testing

The purified mAb or mAb conjugate should be tested for bacterial and fungal bioburden or sterility according to the methods described in Part A, sections 5.1 and 5.2 of the WHO General requirements for the sterility of biological

substances (*57*), or using methods approved by the NRA. Any purified mAb or mAb conjugate substance that is contaminated should be discarded and not subject to re-purification or filtration. If a preservative or other agent has been added to the purified mAb or mAb conjugate, then appropriate measures should be taken to ensure it does not interfere with the tests.

A.5.6.10 Endotoxin

The endotoxin content of each lot of purified mAb or mAb conjugate should be determined and shown to be within limits agreed with the NRA. Suitable in vitro methods include the test for bacterial endotoxins using recombinant factor C or the Limulus amoebocyte lysate (LAL) test. The test used for endotoxin content determination should be validated for its intended purpose.

A.5.6.11 Ratio of payload to mAb (if applicable)

For purified mAb conjugates, the ratio of the payload to mAb (expressed, for example, as g/g or mol/mol) should be calculated. For this ratio to be a suitable marker of conjugation, the quantity and concentration of each of the conjugate components prior to their use should be known. For each purified mAb conjugate, the ratio should be within the range approved by the NRA for that particular conjugate and should be consistent with the ratio in the product shown to be effective in clinical trials.

A.6 Preparation and control of the final bulk

The inclusion of excipients in a mAb product is common. Where included, they must meet their quality specifications and their use, and combination, must be considered safe for human administration at the intended concentrations. The excipients selected and their concentrations in the final bulk should be assessed during the product development process and shown to have no deleterious effects on the mAb or mAb conjugate safety, function, structure or stability – and not to promote mAb aggregation. The types and concentrations of all excipients used should be approved by the NRA.

Appropriate in-process control strategies for the preparation of the final bulk should be established during the product development process and updated as needed throughout its life-cycle. Control strategies should be based on knowledge gained through manufacturing process experience and risk assessment, and must be scientifically justified. Time limits and conditions must be established and validated for the final bulk material prior to filling. Data to support time limits may be generated through small-scale studies, clinical trials or commercial manufacturing experience, and/or from aseptic process simulations. Additional information on the control of the final bulk may be found in relevant ICH guidelines (*8*, *9*, *11*, *69*).

A.6.1 Preparation

The final bulk is prepared by mixing a suitable quantity of the purified bulk mAb or mAb conjugate with all other product constituents, such as buffers, stabilizers, bulking agents, preservatives, other purified bulk mAbs or mAb conjugates, and/or other active pharmaceutical ingredients.

The final bulk should be prepared using a process which has been validated at commercial scale, and should meet the specifications based on the quality attributes of product lots that have been shown to be safe and efficacious in clinical trials. The maximum hold time and storage conditions of the final bulk prior to filling must be validated and supported by data which reflect the stability-indicating attributes.

A.6.2 Test for ratio of combined mAbs (if applicable)

If two or more mAbs and/or mAb conjugates are co-formulated during the preparation of the final bulk, a test must be in place to ensure the proper ratio of the combined mAbs. This test may not be required on the final bulk if the ratio of the combined mAbs is to be verified in the final product (see section A.8.2.7 below).

A.6.3 Bioburden

The final bulk should be controlled for bioburden prior to its sterile filtration. The methods used to control and monitor bioburden must be clearly defined.

A.7 Filling and containers

The relevant guidance provided in WHO good manufacturing practices for pharmaceutical products: main principles (*42*) and WHO good manufacturing practices for biological products (*43*) should be followed.

All containers and container closure systems must be tested for compatibility with the final product formulation and must also be in compliance with NRA requirements, such as those on biological reactivity, leachables and extractables. Similarly, the compatibility of the final product with recommended in-use preparation and administration components (for example, infusion set or filters) should be demonstrated. If any animal-derived materials (for example, colourants made from tallow or fatty acids used in polymer production) are used in the manufacture of the container or closure then assurance of the absence of the prion responsible for TSE (*53*) should be provided. Integrity testing of the containers and closures must be carried out to ensure that they can maintain the stability and sterility of the contents for the duration of the product shelf-life.

Care should be taken to ensure that the materials of which the containers and closures are made (and, if applicable, the transference devices) do not adversely affect the quality of the mAb product. In particular, as mAbs in high

concentration have a propensity to form aggregates, the containers and closures should not induce or otherwise promote aggregation. To this end, a container closure integrity test and assessment of extractables and/or leachables for the final container closure system are generally required for the qualification of containers and may be needed as part of stability assessments.

If multi-dose containers are used and the mAb products do not contain a preservative, then the label and/or Instructions for Use should clearly indicate a time restriction on use following the first withdrawal. In addition, the multi-dose container should prevent microbial contamination of the contents after opening. The manufacturer should also provide the NRA with adequate data demonstrating the stability of the product under appropriate conditions of storage and shipping.

A.8 Control of the final product

A.8.1 Inspection of the final containers

All filled final containers should be inspected as part of the routine manufacturing process. Any containers showing abnormalities (for example, defects or improper sealing) should be discarded. Inspections should be performed against both a black and a white background or using qualified automatic inspection machines, and according to compendial specifications and as recommended by the NRA.

A.8.2 Control tests on the final lot

The following tests should be performed on each lot of mAb, mAb conjugate or co-formulated mAb product using tests validated and approved by the NRA. Due to the complexity of some final containers and closure systems (for example, pre-filled syringes or pens), control testing may need to occur prior to their final assembly. In such cases, a risk assessment should be conducted to determine the appropriate points for control testing. The method development and validation process should include a demonstration that any excipients (for example, preservatives or stabilizers) included in the final formulation do not interfere with the assays. The permissible limits for tests listed under this section should be justified and approved by the NRA, and assay results should support label claims.

Some control tests on the final product may be omitted if they have been conducted on the final bulk prior to filling and if the filling process has been demonstrated not to impact the relevant quality attribute. Similarly, once consistency of production has been demonstrated, it may be possible to omit some tests if sufficient justification is provided. However, changes to the manufacturing process may require the tests to be reinstated until consistency of production has again been verified. The omission of any final product control test should be discussed with, and approved by, the NRA.

A.8.2.1 Appearance

The appearance of the final container and its contents should be verified using a suitable method, and should meet the established criteria with respect to physical state (for example, solid or liquid) and colour, taking into consideration the nature of the container (for example, a dark amber container). The appearance of lyophilized or freeze-dried products should be verified both before and after reconstitution with the intended diluent, and should meet the established criteria.

MAbs are prone to the formation of visible particles, especially at high protein concentrations. Although appropriate formulation development should prevent this from occurring in the final product, the presence of visible particles may not always be avoidable. The specifications regarding appearance would therefore be product specific and may also be dependent on the intended route of mAb product administration. However, any relevant compendial and/or regulatory guidance on visible and subvisible particles must be followed.

A.8.2.2 Identity

Identity tests on the mAb, mAb conjugate or co-formulated mAb product should be performed on each final lot. The identity tests selected should be specific and may be based on the antigen target specificity, molecular structure, isotype, light chain composition and/or other specific properties of the mAb product. More than one identity test may be necessary. For mAb conjugate products, the presence of its conjugated payload must be verified. For co-formulated mAb products, release testing methods should include an identity method that demonstrates the presence of each individual antibody and a quantitative method to confirm their ratio.

A.8.2.3 pH and osmolality

If the mAb product is a liquid preparation, the pH of each lot should be controlled and the results should be within the range approved by the NRA. For a lyophilized preparation, the pH should be measured after reconstitution using the same diluent recommended for clinical use.

The osmolality of the final lots should be determined and shown to be within the range considered to be safe for parenteral administration to humans and agreed with the NRA.

A.8.2.4 Moisture content (if applicable)

If the final product is a lyophilized preparation, the level of residual moisture should be determined and the results should be within the limit agreed with the NRA.

A.8.2.5 Protein content

Protein concentration should be measured using a validated method of suitable sensitivity and specificity – such as determination of the absorbance at 280 nm, using the protein-specific absorbance. The protein concentration of the final product must be within ± 10% of the labelled claim. For co-formulated mAb products, the protein content of each of the individual mAbs should be measured.

A.8.2.6 Potency

Potency testing should be conducted for each final product lot. As outlined in section A.5.6.5 above, the test method(s) used should reflect the activity/activities of the mAb or mAb conjugate. Potency should be expressed as a value relative to a reference material, and the assay should be sufficiently sensitive to detect functional differences in the product. Both the method of analysis and selection of the reference material should be approved by the NRA. Any potential effect(s) on the potency assay(s) caused by excipients contained in the product formulation should be considered.

For co-formulated mAbs, the potency methods used should account for all mAb substances present in the final product.

In rare cases, animal-based assays may be required to assess the potency of certain mAbs. However, such assays are notoriously variable and difficult to validate, and should not be used when an in vitro alternative is available (see section A.5.6.5 above). The use of animal methods should in all cases adhere to the 3Rs principles ("Replace, Reduce, Refine") to minimize the use of animals in testing, and should be discussed with the NRA.

A.8.2.7 Test for ratio of combined mAbs (if applicable)

If two or more mAbs and/or mAb conjugates are co-formulated in the final product, a test must be in place to ensure the proper ratio of the combined mAbs. This test may not be required on the final product if the ratio of the combined mAbs was verified in the final bulk (see section A.6.2 above).

A.8.2.8 Heterogeneity profile

The heterogeneity profile of the final product should be confirmed as being similar to that of the purified mAb substance (see section A.5.6.6 above). Some differences in the heterogeneity profile might occur during substance storage and final product manufacturing (for example, formation of aggregates) and should be justified in such cases. Attributes which should be considered during final product consistency assessment include the size distribution, charge heterogeneity and other post-translational modifications. Conjugated mAbs should also be verified in terms of the heterogeneity of the payload-to-mAb ratio. The number

of methods used to assess heterogeneity may be reduced if the impact of the formulation and filling processes are clearly characterized and demonstrated to have little effect – however, this should be appropriately justified and discussed with the NRA. The measurement of some product-related post-translational modifications in the drug substance may be sufficient and not require further retesting if the drug product manufacturing process is demonstrated to not have an impact on the post-translational modifications.

A.8.2.9 Product-related impurities

Protein isoforms identified as product-related impurities should be measured in the final product. Some product-related impurities may only need to be measured in the drug substance if the downstream drug product manufacturing process is demonstrated to not have an impact on those impurities. HPLC (for mAb and mAb conjugates) and capillary electrophoresis or SDS-PAGE (for mAb fragments) are common methods of choice for quantitating such impurities, though other techniques may also be used.

As mAbs are susceptible to aggregation, each final lot should be examined for particulate matter and aggregate content at lot release and at the end of its shelf-life unless it can be shown that this is not necessary. Fragmentation patterns should also be assessed in the final lot and at the end of its shelf-life and compared to the historical data for that same product.

A.8.2.10 Process-related impurities

Measurement of process-related impurities (for example, elemental and nitrosamine impurities, and impurities from excipients or bulking agents, the container closure system or from other potential sources during the preparation of the final bulk and during the filling process) should be considered. If not measured in the final product, the control of process-related impurities should be demonstrated. If clearance of process-related impurities has been demonstrated, or the impurity is controlled as an in-process control or tested in the final bulk, there may be justification for excluding it as a release test in the final lot.

For products containing mAb conjugate(s), a limit should be set for an acceptable amount of unbound (free) payload(s). The acceptable limit should be consistent with the value seen in batches used for clinical trials that showed adequate activity, and should be approved by the NRA.

A.8.2.11 Excipients

The presence and concentration of excipients critical to product stability and sterility (such as surfactants or preservatives) should be controlled. With the

exception of compendial grade excipients, testing requirements for all excipients should be based on risk assessment and discussed with the NRA.

A.8.2.12 Sterility

The contents of the final containers should be tested for bacterial and fungal sterility according to the methods described in Part A, sections 5.1 and 5.2 of the WHO General requirements for the sterility of biological substances (*57*), or using a method approved by the NRA. If the final product contains a preservative, then appropriate measures should be taken to prevent it from interfering with the tests.

A.8.2.13 Endotoxin or pyrogen content

The endotoxin content of each lot of the final product should be consistent with levels found to be acceptable in product lots used during clinical trials. Suitable in vitro methods include the test for bacterial endotoxins using recombinant factor C or the LAL test. The test selected for assessing endotoxin content must be validated for its intended purpose.

Many NRAs expect a parenterally administered drug product to have an endotoxin content of ≤ 5 EU/kg/h in its final presentation, or ≤ 0.2 EU/kg/h for intrathecally administered products. Therefore, the potential contribution of endotoxin from a reconstitution buffer, diluent or other co-administered product should also be considered.

The need for pyrogenicity testing should be determined during the manufacturing development process based on an appropriate risk assessment. This may need to be re-evaluated following any changes in the production process or relevant reported production inconsistencies that could influence the quality of the product with regard to its pyrogenicity. A monocyte activation test may be used for monitoring the potential pyrogenic activity in the final product after a product-specific validation. Although a rabbit pyrogenicity test may be accepted by the NRA, its use is discouraged due to the inherent variability, high re-testing rates and interspecies differences in pyrogenic responses compared to humans.

A.8.2.14 Reconstitution time (if applicable)

The reconstitution time should conform to specification if the final product is presented as a freeze-dried or lyophilized formulation.

A.8.2.15 Extractable volume

It should be demonstrated that the nominal volume indicated on the label can consistently be extracted from the containers, whether single-dose or multi-dose.

A.9 Records

The relevant guidance provided in WHO good manufacturing practices for pharmaceutical products: main principles (*42*) and in section 17 of WHO good manufacturing practices for biological products (*43*) should be followed as appropriate for the level of development of the product. Written records should be kept of all tests, irrespective of their results. The records should be of a type that allows for any trends in critical parameters and release testing to be monitored.

A.10 Retained samples

The guidance provided in section 16 of WHO good manufacturing practices for biological products (*43*) should apply. A sufficient number of samples from each lot of the product should be retained for future studies and needs. MAb, mAb conjugate or co-formulated mAb product lots that are to be used for clinical trials may serve as reference materials in the future and a sufficient number of final containers should be reserved and appropriately stored for that purpose.

A.11 Labelling

The guidance on labelling provided in section 14 of WHO good manufacturing practices for biological products (*43*) should be followed as appropriate. Labelling should also conform to the national requirements of the country in which the product will be used. For clinical trial samples, labelling requirements vary by country and the NRA should be consulted. For marketed products, all claims on the product label must be met by the lot release tests detailed in section A.8.2 above. In addition, the label on the carton, container and/or leaflet accompanying each container, should include:

- the product name, INN and lot number of the mAb product;
- the volume of one recommended human dose, and the recommended schedule and route(s) of administration;
- the amount of active substance(s) contained in one human dose;
- the number of doses if the product is issued in a multi-dose container, and the storage conditions and shelf-life after opening;
- the name and concentration of any antibiotic, preservative and/or excipient added;
- the temperature recommended during storage and transport;
- the expiry date;
- contraindications, warnings and precautions, concomitant product use advice, and potential adverse reactions; and

- if applicable, information on the volume and nature of the diluent to be added to reconstitute a powdered or lyophilized product; the instruction that any product in a lyophilized form should be used immediately after reconstitution or the approved duration of storage of the reconstituted product.

For mAb products co-formulated with multiple active substances, the total dose of the product as well as the amount of the individual substances within that dose should be indicated.

A.12 Distribution and transport

The guidance provided in WHO good manufacturing practices for pharmaceutical products: main principles (*42*) and WHO good manufacturing practices for biological products (*43*) should be followed. Shipping studies from drug substance to drug product manufacturing sites (if at different locations) and release to in-country distributor(s) should be conducted and validated for the commercial supply chain.

Shipments should be maintained within specified temperature ranges, and packages should contain cold-chain monitors if the temperature needs to be controlled. If it is claimed that a cold chain is not required then the conditions under which stability has been established (for example, maximum temperature and maximum length of time at that temperature) should be described and data supporting these claims provided. Further guidance on these and related issues is provided in WHO Model guidance for the storage and transport of time- and temperature-sensitive pharmaceutical products (*76*).

A.13 Stability testing, storage and expiry date

A.13.1 Stability testing

Stability programmes for intermediates, drug substances and drug products should be initiated early in the development process. When relevant, in-use stability studies should be conducted to establish the time period during which a drug product may be used after the container is opened while still retaining acceptable quality specifications.

Stability study protocols and results supporting the stability claims over the shelf-life must be provided to the NRA. Recommended storage conditions for drug substances and products should be based on the stability data. Stability programmes for lyophilized products should be conducted following reconstitution with the intended diluent. Appropriate studies should be considered for multi-dose containers to demonstrate maintenance of product quality and microbial control during the in-use period.

Appropriate stability-indicating parameters should be defined or selected according to the stage of production. When changes are made in the production procedure that may affect the stability of the product, further stability studies may need to be conducted to determine the validity period of the new product as per the WHO Guidelines on procedures and data requirements for changes to approved biotherapeutic products (*45*).

For radio-labelled mAbs, stability studies may be conducted using non-radioactive labels and limited to the expected duration over which the radioisotope is considered to be active.

Long-term stability studies for final products are required. Stability studies under accelerated and stress conditions are strongly advised in WHO (*2*) and ICH (*77*) guidelines and may be required by NRAs for a marketing application. Such studies provide additional information on the overall characteristics of the mAb substance(s) and product, and help identify stability-indicating methods suitable for ongoing stability studies. This information may also be useful in assessing comparability should the manufacturer plan to make future changes to the manufacturing process.

For mAb product licensure, the stability and expiry date of the product in its final container, when maintained at the recommended storage temperature should be demonstrated to the satisfaction of the NRA using final containers from at least three final lots made from different mAb bulks. For products filled in more complex containers (for example, in a device) stability testing might be considered after the final container closure is secured but prior to the addition of non-container-closure parts. Although fewer data are likely to be available during clinical trials, the stability of the mAb product under the proposed storage conditions should be demonstrated for at least the expected duration of the clinical trial.

Following licensure, ongoing monitoring of mAb product stability will be required to support shelf-life specifications and to refine the stability profile. Data should be provided to the NRA according to local regulatory requirements.

The final stability-testing programme should be approved by the NRA and should include an agreed set of stability-indicating parameters, as well as procedures for the ongoing collection and sharing of stability data. In-use stability and, where applicable, compatibility (for example with infusion sets) should also be specified and justified with adequate data generated under real-time conditions.

The use of stability data obtained while the mAb product is in clinical trials may be considered under certain circumstances, and when there is an urgent need to reduce product development time. Any knowledge gained regarding the stability of other mAbs which differ only in their antigen-binding domain and which have been manufactured using the same platform technology may also provide valuable insight into the stability of novel products. However, the NRA should be consulted regarding the use of either approach.

A.13.2 Storage conditions

Storage conditions should be well defined and fully validated. The mAb product should have been shown under these conditions to maintain its potency for a period equal to that from the date of release to the expiry date. During clinical trials, the stability of the mAb product under the proposed storage conditions should be demonstrated for at least the expected duration of the clinical trial in accordance with guidance on the extrapolation of stability for products in development (*78*).

A.13.3 Expiry date

The expiry date should be based on the shelf-life and should be approved by the NRA. Relevant guidance on calculation methods for expiry periods can be found in section 8.3 of the WHO Guidelines on stability evaluation of vaccines (*79*).

Part B. Guidelines for NRAs

B.1 General

MAbs represent a broad spectrum of products and manufacturing processes and it is therefore not possible to establish a fixed set of attributes or testing methods that would necessarily apply to all of them. Therefore, it is advised that each product and manufacturing process be assessed on a case-by-case basis with some provision for flexibility to allow for the introduction of innovative approaches based on sound scientific principles and practices.

The guidance for NRAs and national control laboratories (NCLs) given in the WHO Guidelines for national authorities on quality assurance for biological products (*47*) should be followed. These guidelines specify that no new biological product should be released until consistency of lot manufacturing and quality has been established and demonstrated by the manufacturer.

MAb manufacturing processes and control strategies should be described in detail in the dossier of the marketing application. Any subsequent manufacturing and/or control strategy changes must be assessed for their potential impact on product quality, safety and efficacy using a risk-based approach and in accordance with the WHO Guidelines on procedures and data requirements for changes to approved biotherapeutic products (*45*). Such changes may require assessment and approval by the NRA. For control purposes, any relevant international reference materials currently in force should be obtained for the purpose of calibrating national, regional and working standards as appropriate.

Independent lot release by NRAs or NCLs of mAb products may apply in some countries. The release process or testing strategies used in each country should follow a risk-based approach in line with WHO Good reliance practices in

the regulation of medical products: high level principles and considerations (*80*). The NRA and/or NCL may obtain from the manufacturer the product-specific and/or working references and reagents for testing purposes.

Consistency of production is an essential component in the quality assurance of mAb products. The NRA should monitor production records and quality control test results for clinical lots, as well as for a series of consecutive lots of the final bulk (that is, the drug substance) and/or final product.

In addition, the NRA should satisfy itself with regard to the data gathered for ensuring therapeutic effect and safety in humans, and evaluate for its approval:

- all methods used in the manufacture of mAb products;
- the criteria used to establish the manufacturer's reference materials;
- all tests for extraneous agents and for total protein;
- all tests for preservatives and for agents used for purification or during other stages of manufacture;
- the tests used to determine the distribution of molecular size;
- the tests used to determine the potency of the mAb, and define the acceptable range of estimated mean values and the fiducial limits;
- the dose to be administered;
- the concentration of preservative and excipients in the final product, if added;
- the purity of the final product; and
- the statements concerning storage temperature and expiry date appearing on the label.

The NRA should satisfy itself that the results of all tests, including those conducted to validate the manufacturing process, are satisfactory and that consistency of production and testing have been established.

B.2 Official release

Products containing mAbs should only be released if they fulfil all national requirements and/or satisfy Part A of these WHO Guidelines.

Authors and acknowledgements

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Dr S. Hufton, National Institute for Biological Standards and Control, the United Kingdom and Dr J. Siggers, Health Canada, Canada. The General considerations section was drafted by Dr I. Feavers, consultant, Nacton, the United Kingdom.

The resulting draft document was then posted on the WHO Biologicals website from 18 October to 30 November 2021 for a first round of public consultation. Comments were received from: Mr R.M. Bretas, Agência Nacional de Vigilância Sanitária, Brazil; Dr M. Buda, European Directorate for the Quality of Medicines & Healthcare, France; Dr R.M. Dhere, Serum Institute of India Pvt. Ltd., India; Dr M. Gencoglu, International Federation of Pharmaceutical Manufacturers & Associations (IFPMA), Switzerland; Dr O. Kwon, Ministry of Food and Drug Safety, Republic of Korea; Ms L.J. Rodríguez and Ms A. Valdés-Alanís, PROBIOMED S.A. de C.V., Mexico; Ms E. Satterwhite and the IGBA Biosimilars Committee, International Generic and Biosimilar Medicines Association (IGBA), Switzerland; Dr J. Siggers, Health Canada, Canada; Dr J. Southern, Advisor to the South African Health Products Regulatory Authority, South Africa; and Dr R.M. van der Plas, Medicines Evaluation Board, Netherlands.

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Appendix 1

Examples of mAb expression systems used in the manufacture of marketed mAb products or under evaluation and development for such a purpose

Expression system type	Examples
In vivo	Mouse ascites
Mammalian cell lines	NS0, CHO, BHK, HEK 263, HKB-11, PER.C6
Prokaryotic cells	*Escherichia coli*, *Proteus mirabilis*, *Pseudomonas putidas*, Bacillus/ Lactobacillus species
Eukaryotic cells:	
Yeast	*Pichia pastoris*, *Saccharomyces cerevisiae*, *Hansenula polymorpha*
Fungi	Trichoderma species, Aspergillus species
Protozoa	*Leishmania tarentolae*
Insects	*Spodoptera frugiperda*, *Drosophila melanogaster*, *Trichopulsia ni*
Plants	Nicotiana species, *Lemna minor*, *Arabidopsis thaliana*, *Medicago sativa*
Transgenic animals	Milk expressed from mice or goats, chicken eggs
Emerging technology	In vitro cell-free synthesis

Appendix 2

Summary of potential sources of heterogeneity in recombinant mAbs and examples of possible characterization methods[22]

[22] CE-SDS = capillary electrophoresis with sodium dodecylsulfate; LC-MS = liquid chromatography–mass spectrometry; HPAEC-PAD = high-performance anion-exchange chromatography with pulsed amperometric detection.

Table 1

Heterogeneity	Physicochemical change	Suggested methods of analysis	Comments
Primary structure	Amino acid sequence variation	Deduced from nucleotide sequence and can be supported by results from peptide LC-MS	The primary amino acid sequence should be compared against the predicted sequence determined by whole genome or deep sequencing master and working cell banks
N- and C- terminal modifications	Mass and charge	Ion exchange chromatography Isoelectric focusing Capillary electrophoresis Peptide LC-MS	Detection methods can be combined with mass spectrometry for detailed identification
Glycosylation	Mass and charge	N-glycan release by PNGase F followed by hydrophilic interaction chromatography or capillary electrophoresis	Detection by fluorescence or mass spectrometry does not provide site-specific information
		HPAEC-PAD	
		LC-MS	Peptide level MS required for site-specific data
Glycation	Mass and charge	LC-MS	Peptide level MS required for site-specific data

Table 1 *continued*

Heterogeneity	Physicochemical change	Suggested methods of analysis	Comments
Hydrogen bond modifications			
Alternative disulphide linkages	Charge	Peptide LC-MS under reducing and non-reducing conditions	
Free sulphydryl groups	Mass, charge and hydrophobicity		
Trisulphide bonding	Mass and charge		
Formation of thioether	Mass		
Amino acid modifications			
Asn deamidation	Mass and charge	LC-MS	Deamidation can be an artefact of sample preparation for LC-MS
Asp isomerization	Charge and hydrophobicity	LC and peptide LC-MS	
Succinimide	Mass, charge and hydrophobicity	LC-MS	
Oxidation	Mass and hydrophobicity	Reverse phase chromatography, peptide LC-MS	
Molecular size species (aggregates and fragments)	Mass, visible and subvisible particle formation	Reduced and non-reduced CE-SDS Size-exclusion chromatography without or with multiangle light scattering Light obscuration Nanoparticle tracking Microflow imaging Analytical ultracentrifugation	Due to potentially wide size range may need multiple methods

Annex 5

New and replacement WHO international reference standards for biological products

The provision of global measurement standards is a core normative WHO activity. WHO international reference standards are widely used by manufacturers, regulatory authorities and academic researchers in the development and evaluation of biological products. The timely development of new reference standards is crucial in harnessing the benefits of scientific advances in new biologicals and in vitro diagnosis. At the same time, management of the existing inventory of WHO international reference standards requires an active and carefully planned programme of work to replace established materials before existing stocks are exhausted.

The considerations and guiding principles used to assign priorities and develop the programme of work in this area have previously been set out as WHO Recommendations.[23] In order to facilitate and improve transparency in the priority-setting process, a simple tool was developed as Appendix 1 of these WHO Recommendations. This tool describes the key considerations taken into account when assigning priorities, and allows stakeholders to review and comment on any new proposals being considered for endorsement by the WHO Expert Committee on Biological Standardization.

A list of current WHO international reference standards for biological products is available at: https://www.who.int/teams/health-product-and-policy-standards/standards-and-specifications/catalogue.

At its meetings held via video conference on 4–8 April 2022, the WHO Expert Committee on Biological Standardization made the changes shown below to the previous list. Each of the WHO international reference standards shown in this table should be used in accordance with their instructions for use (IFU).

[23] Recommendations for the preparation, characterization and establishment of international and other biological reference standards (revised 2004). In: WHO Expert Committee on Biological Standardization: fifty-fifth report. Geneva: World Health Organization; 2006: Annex 2 (WHO Technical Report Series, No. 932; https://www.who.int/publications/m/item/annex2-trs932).

Additions[24]

Material	Unitage	Status
In vitro diagnostics		
Anti-human neutrophil antigen-3a immunoglobulin G	1 in 8 dilution assigned as the minimum potency	WHO International Reference Reagent
Lassa virus RNA for NAT-based assays	4.0 $\log_{10}$ IU/ampoule	First WHO International Standard
Anti-β2GPI immunoglobulin G	200 IU/vial	First WHO International Standard
Standards for use in high-throughput sequencing technologies		
Gut microbiome	No unitage assigned	WHO international reference reagents
Vaccines and related substances		
Anti-enterovirus D68 serum (human)	1000 IU/ampoule	First WHO International Standard

[24] Unless otherwise indicated, all materials are held and distributed by the National Institute for Biological Standards and Control, Potters Bar, Herts, EN6 3QG, the United Kingdom.

SELECTED WHO PUBLICATIONS OF RELATED INTEREST

WHO Expert Committee on Biological Standardization
Seventy-fourth report.
WHO Technical Report Series, No. 1039, 2022 (xv + 157 pages)

WHO Expert Committee on Biological Standardization
Report of the seventy-second and seventy-third meetings.
WHO Technical Report Series, No. 1030, 2021 (xvii + 269 pages)

WHO Expert Committee on Biological Standardization
Seventy-first report.
WHO Technical Report Series, 1028, 2021 (xii + 102 pages)

WHO Expert Committee on Biological Standardization
Seventieth report.
WHO Technical Report Series, No. 1024, 2020 (xvi + 227 pages)

WHO Expert Committee on Biological Standardization
Sixty-ninth report.
WHO Technical Report Series, No. 1016, 2019 (xv + 251 pages)

WHO Expert Committee on Biological Standardization
Sixty-eighth report.
WHO Technical Report Series, No. 1011, 2018 (xvi + 380 pages)

WHO Expert Committee on Biological Standardization
Sixty-seventh report.
WHO Technical Report Series, No. 1004, 2017 (xviii + 591 pages)

WHO Expert Committee on Biological Standardization
Sixty-sixth report.
WHO Technical Report Series, No. 999, 2016 (xix + 267 pages)

WHO Expert Committee on Biological Standardization
Sixty-fifth report.
WHO Technical Report Series, No. 993, 2015 (xvi + 262 pages)

Website: https://www.who.int/health-topics/Biologicals#tab=tab_1

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This report presents the recommendations of a WHO Expert Committee commissioned to coordinate activities leading to the adoption of international recommendations for the production and control of vaccines and other biological products used in medicine, and the establishment of international biological reference materials.

Following a brief introduction, the report summarizes a number of issues brought to the attention of the Committee at its meeting held virtually in April 2022. Of particular relevance to manufacturers and national regulatory authorities are the discussions held on the development and adoption of new and revised WHO Recommendations, Guidelines and guidance documents. Following these discussions, the following three documents were adopted on the recommendation of the Committee: (a) WHO manual for the preparation of reference materials for use as secondary standards in antibody testing; (b) Guidelines on evaluation of biosimilars; and (c) Guidelines for the production and quality control of monoclonal antibodies and related products intended for medicinal use.

Subsequent sections of the report provide information on the current status, proposed development and establishment of international reference materials in the areas of: cell and gene therapy products; in vitro diagnostics; standards for use in high-throughput sequencing technologies; standards for use in public health emergencies; and vaccines and related substances.

A series of annexes is then presented which includes an updated list of all WHO Recommendations, Guidelines and other documents related to the manufacture, quality control and evaluation of biological products (Annex 1). The above three WHO documents adopted on the advice of the Committee are then presented as part of this report (Annexes 2–4). Finally, all new and replacement WHO international reference standards for biological products established during the April 2022 meeting are summarized in Annex 5. The updated full online catalogue of WHO international reference standards is available at: https://www.who.int/teams/health-product-and-policy-standards/standards-and-specifications/catalogue.

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