# Guidelines on the replacement or removal of animal tests for the quality control of biological products

**Adopted on the recommendation of the Eighty-first meeting of the World Health Organization Expert Committee on Biological Standardization, 13–16 October 2025. This finalized and edited document is being provided here prior to its formal publication both as a standalone resource in the WHO Institutional Repository for Information Sharing (IRIS) and as Annex 2 of the full report of the above meeting which will appear in the WHO Technical Report Series**.

# Annex 2

## Guidelines on the replacement or removal of animal tests for the quality control of biological products

**Important note**

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, the guidance given in these WHO Guidelines may be adopted as definitive national requirements, or modifications may be justified and made by the NRA. It is recommended that such modifications are made only on condition that any final product is at least as safe and efficacious as that prepared in accordance with the guidance set out below.

The implementation of existing in vitro alternatives to animal-based testing for the quality control of biological products and the development of new in vitro methods, particularly where gaps still remain, are strongly recommended. Towards this goal, international cooperation between relevant stakeholders, along with enhanced regulatory convergence, will be vital in accelerating the global acceptance of fit-for-purpose in vitro tests for the quality control of biological products.

The guidance provided in the current document with regard to the use of in vitro tests, and by extension the replacement or removal of animal tests, is science-based. Such guidance should be viewed as superseding the corresponding quality control recommendations specified in WHO Recommendations, Guidelines and other guidance documents on biological products published prior to 2025. Product developers and manufacturers, and other stakeholders should not await the updating of these previously published WHO documents but should instead, wherever possible, develop, validate and implement non-animal-based in vitro approaches to the quality control of biological products in close consultation with, and the approval of, the NRA.

## Abbreviations

| | |
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| CHO | Chinese hamster ovary |
| CNS | central nervous system |
| CQA | critical quality attribute |
| ELISA | enzyme-linked immunosorbent assay |
| EPO | Erythropoietin |
| FNV | French neurotropic vaccine |
| GMP | good manufacturing practices |
| HTS | high-throughput sequencing |
| ICH | International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use |
| LAL | *Limulus* amoebocyte lysate |
| MAPREC | mutant analysis by PCR and restriction enzyme cleavage |
| MAT | monocyte activation test |
| MNVT | monkey neurovirulence test |
| mRNA | messenger RNA |
| NC3Rs | National Centre for the Replacement, Refinement and Reduction of Animals in Research |
| NCL | national control laboratory |
| nOPV2 | novel OPV type 2 |
| NRA | national regulatory authority |
| OPV | oral poliomyelitis vaccine |
| PBMC | peripheral blood mononuclear cell(s) |
| PCR | polymerase chain reaction |
| rCR | recombinant Cascade Reagent |
| rFC | recombinant Factor C |
| RNA | ribonucleic acid |
| RPT | rabbit pyrogen test |
| TAL | *Tachypleus* amoebocyte lysate |
| TgmNVT | transgenic mouse neurovirulence test |

## 1. Introduction

Animal testing has long been an important tool in the development of medicinal biological products, providing critical information on their mechanisms of action, safety and efficacy. In some cases, such testing also continues to be used post-approval to monitor product quality as part of the quality control processes of manufacturers and national control laboratories (NCLs). However, the growing recognition that appropriate in vitro tests are better suited for routine quality control of biological products has become a key driver of a shift towards quality control schemes that avoid the use of animals altogether. As a result, major advances are now being made in the development and implementation of non-animal methods for the quality control of biological products, driven and guided by scientific evidence and technological innovations.

Historically, efforts to conduct high-quality scientific research in the most humane way have been underpinned by the guiding principles of the "3Rs" approach of replacement, reduction and refinement *(1)*. This approach aims to promote good practices and the humane treatment of animals used in scientific research and product testing, including through reduction of the number of animals used and refinement of animal test methodologies. However, while reduction and refinement efforts may help to promote animal welfare, such approaches (in particular, reduction) can increase the variability of test results. In contrast, the replacement of animal tests with appropriate in vitro tests eliminates any requirement to use animals while maintaining or improving the scientific relevance of the data obtained. The use of in vitro assays can substantially reduce assay variability, as well as the time and resources required, thereby improving the predictability and timely release of safe and efficacious biological products *(2)*. Where the replacement or removal of animal tests from the quality control process is not immediately possible, rationalized and streamlined testing strategies should be used in which an animal test is performed only once at a crucial manufacturing step, or for a limited number of lots manufactured during process development and/or during commercial manufacturing, in order to render further such testing unnecessary. In all cases, where the replacement or removal of animal tests based on the above strategies is not yet feasible, efforts should be made to develop, validate and implement suitable in vitro approaches *(3–6)*.

For more than 70 years, WHO Recommendations, Guidelines and other guidance documents have set the norms and standards for the production, quality control, and nonclinical and clinical evaluation of biological products. Based on scientific consensus achieved through extensive international collaboration and consultation, these global norms and standards support efforts by countries to ensure the quality, safety and efficacy of licensed biological products. While acknowledging that animal-based research currently remains integral to the development of many such products, WHO also recognizes the scientific limitations of many of the animal assays still appearing in some of its published guidance on biological products, particularly with regard to their use in post-approval product quality control.

In light of technological advances and resulting opportunities for the improved quality control of biological products based on the development and implementation of scientifically sound and reproducible non-animal methods, a need was recognized to align WHO guidance with current thinking among manufacturers and regulators worldwide. In 2019, the WHO Expert Committee on Biological Standardization endorsed a proposal to commission an

independent review of all animal testing requirements in WHO Recommendations, Guidelines and other guidance documents. This review was carried out by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) in the United Kingdom, and was co-funded by the Gates Foundation *(7)*. The review aimed to identify all of the animal tests recommended by WHO for biological product quality control, and to highlight the opportunities for, and barriers to, the implementation of alternative non-animal tests. The project was overseen by an international expert working group of regulators, manufacturers and other relevant stakeholders and its final report was presented to the Committee in October 2023 *(8–10)*.

Of the 81 WHO documents reviewed, 63 included animal test methods used to assess, for example, the presence of adventitious agents, neurovirulence, potency, pyrogenicity and specific toxicity. In each case, the guidance was reviewed and alternative wording proposed to provide clearer and more standardized language *(8)*. In addition, the expert working group made several further recommendations, including that WHO prepare a position statement and provide specific guidance in this area based on sound scientific principles. WHO also received requests from other stakeholders with an interest in implementing in vitro approaches for the quality control of biological products. Recognizing the challenge of revising each of the WHO documents individually, the Committee instead recommended that standalone science-based WHO guidance be developed on the replacement or removal of animal tests still used in the quality control of biological products. The current document was therefore developed through an extensive consultation process *(11)* to provide guidance on the implementation of in vitro methods to replace the animal-based quality control tests historically recommended in WHO guidance on biological products published prior to 2025, as well as on the potential complete removal of such animal tests.

WHO strongly encourages developers, manufacturers and regulators of medicinal biological products to replace or remove animal-based quality control methods whenever scientifically justified. Statements to this effect have previously been made in both product-specific and more general published WHO guidance on biological products *(12, 13)*, most recently in relation to the recommended discontinuation of the innocuity test *(14, 15)*.

## 2. Purpose and scope

These WHO Guidelines provide guidance to biological product developers, manufacturers and regulators on a range of scientific and regulatory considerations with regard to the replacement or removal of animal tests for the quality control of biological products. The document should be read in conjunction with other relevant WHO guidance, including both product-specific guidance documents *(16)* and more general documents, for example on lot release *(13)*. However, it should be noted that the recommendations provided below in each of the main sections of the current document are intended to supersede any corresponding quality control requirements concerning animal-based assays specified in WHO Recommendations, Guidelines and other guidance documents published prior to 2025.

Consideration of the nonclinical development of biological products, and of disease-specific animal models not currently used in routine quality control, are beyond the scope of this document. However, developers are encouraged to explore opportunities for the

implementation of non-animal models in this context. Where animal-based methods for the routine quality control testing of biological products cannot yet be replaced or removed, efforts should be made to reduce or refine the use of such methods as far as scientifically justified. In all cases, efforts should also be made to develop, validate and implement suitable in vitro approaches.

Although examples are provided in which the comparing of a non-animal method with an existing animal method to establish a correlation is challenging or not scientifically justified, no attempt has been made to include detailed discussion of assay validation itself, the principles of which are comprehensively described elsewhere *(17–22)*.

## 3. Terminology

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

**Adventitious agents** (also called "extraneous agents"): contaminating microorganisms of the cell culture or starting/raw materials, including bacteria, fungi, mycoplasmas/spiroplasmas, mycobacteria, *Rickettsia*, protozoa, parasites, transmissible spongiform encephalopathy agents and viruses, that have been unintentionally introduced into the manufacturing process of a biological product.

**High-throughput sequencing** (also known as massively parallel, next generation or deep sequencing): technology based on sequencing multiple nucleic acid molecules in parallel, thereby increasing sequencing speed and efficiency compared to earlier sequencing methods.

**Neurotropism**: the ability of a virus to invade and replicate in neural tissue.

**Neurovirulence**: the ability of a virus to cause damage to the central nervous system.

**Potency**: the measure of biological activity, using a suitable quantitative assay, based on the attribute of the product that is linked to the relevant biological properties.

**Pyrogenicity**: the ability of a substance to cause an increase in body temperature in a recipient organism.

**Reduction**: the use of appropriately designed and analysed animal experiments that reduce the number of animals used as far as possible while ensuring that the test results remain robust and reproducible.

**Refinement**: improving laboratory animal welfare by using the most up-to-date technologies, and housing, enrichment and handling practices, to minimize pain, suffering and distress.

**Removal**: removing an animal test previously used for quality control purposes based on the assurances provided through the implementation of GMP, use of validated manufacturing processes and appropriate quality control measures and strategies during the manufacturing of biological products.

**Replacement**: replacing an animal test with a non-animal test through the development and use of predictive and robust non-animal models and tools based on current scientific knowledge and technologies.

**Viscerotropism**: the ability of a virus to invade and damage internal organs, particularly of the visceral cavity.

## 4. General considerations

The purpose of tests used within the overall control strategy for the routine quality control of biological products is to ensure that the quality characteristics of commercial lots are consistent with those shown to be safe and efficacious in clinical studies, and to monitor production consistency *(22–29)*. Although animal tests have long played a critical role in ensuring the quality of biological products, their inherent variability and poor precision make them less suitable than well-designed in vitro assays for monitoring production consistency or assessing the potential consequences of manufacturing changes *(2, 30)*. It is therefore important to continually review the scientific value and relevance of such in vivo tests. Those found to be of limited or no value should be removed, and those deemed to be of value should be replaced with suitable non-animal alternatives. Notable progress in the development and implementation of in vitro methods as alternatives to animal tests has resulted in their successful introduction into regulatory requirements and guidelines worldwide *(31)*.

While animal tests may be needed during the development phase of certain products, it is imperative that consideration is also given at the outset to the use of in vitro tests when developing or revising quality control schemes. For example, in vivo potency tests may be used to assess complex functional responses potentially useful for proof-of-concept studies, but these are not necessarily predictive of the actual responses in the target population. By contrast, in vitro assays can measure specific parameters that reflect elements of the complex in vivo responses with lower variability and higher sensitivity *(30)*. Careful product and process characterization and understanding of the critical quality attributes (CQAs) relating to safety and efficacy can thus allow control strategies for the product life-cycle to be designed without any requirement for animal tests. During product development, manufacturers should critically evaluate any potential need for future in vivo tests and consider establishing and validating a test or set of tests to monitor product consistency without the use of animals. Incorporating such in vitro tests as early as possible into the product development process will allow for evaluation of the comparability of clinical materials and subsequent commercial lots.

A systematic approach to product development begins with predefined objectives and emphasizes product and process understanding and process control based on sound science and quality risk management *(23, 24)*. The application of such an approach requires a thorough understanding of the product characteristics and manufacturing processes. The CQAs of the drug substance or drug product are identified in a quality target product profile which can be used to define a control strategy based on a consistent manufacturing process that is carefully monitored within a quality system *(32)*. Such an approach to routine quality control testing should be based on ensuring that the product quality profile stays within the desired range to ensure safety and efficacy. Non-animal methods can be used to monitor quality parameters more precisely than is possible using animal tests, and are therefore better suited to this objective *(5, 33)*. The consistency of quality attributes is the goal, not necessarily replicating a complex and variable in vivo response. Such an approach directly supports the rationale underlying the use of scientifically relevant in vitro methods.

Many biological products, particularly those that have been recently developed, are derived from highly purified components using chemically defined media that avoid all animal-derived supplements to ensure consistency. In addition, significant technological advances

have allowed for the precise characterization of product CQAs using specific and sensitive in vitro assays. As a result, newer biological products have typically been developed using only in vitro methods for the control of their CQAs. Wherever possible, chemically defined, animal-free media that avoid all animal-derived supplements should be used for optimal definition and reproducibility.

In contrast, many legacy products continue to be controlled using in vivo tests dating back to their original approval. The implementation of new analytical approaches for the control of such legacy products has sometimes been delayed due to regulatory challenges. For example, variations to existing marketing authorizations typically require significant commitment of resources, while some regulators have been reluctant to accept innovative approaches to quality control testing despite sound scientific rationales supporting the replacement or removal of in vivo tests *(3)*. Further guidance to NRAs on regulatory acceptance of alternative in vitro approaches that have been adequately validated and scientifically justified, including through the use of reliance-based approaches, is provided in section 12 below. Manufacturers should critically assess the need for the continued use of in vivo tests for the quality control of licensed products, and are encouraged to develop alternative animal-free assays in close dialogue with the NRA.

The inherent high variability of in vivo assays can also present a challenge for their replacement with more consistent in vitro methods. For example, direct assay comparisons are typically expected as part of method replacement – however, in the case of in vivo to in vitro method replacement such comparison studies may not generate meaningful outcomes, due in part to the high variability of in vivo methods. As a result, individual initiatives and international collaborative studies have often failed to demonstrate a correlation between the animal and non-animal methods, independent of the suitability of the in vitro method under study. Furthermore, while many legacy in vivo quality control tests were broadly considered to be fit for purpose, they were adopted in the era prior to the establishment of validation requirements such as those provided in the WHO Good manufacturing practices: guidelines on validation, the ICH Q2(R2) guideline and VICH GL2 validation methodology *(17, 18, 34)*, all of which require the establishment of method precision, reproducibility, limits of detection and quantification. The lack of such defined characteristics for legacy in vivo assays constrains method comparison. Importantly, when an in vivo test is replaced with an in vitro alternative, the CQAs of the product are typically assessed differently (for example, determination of antigen content and quality instead of in vivo potency, or a cell-based toxicity assay instead of an animal toxicity test). Therefore, demonstrating correlation between the two methods is generally not scientifically justified and should not be expected. Even where the pass/fail outcomes from the two test procedures are in agreement, the degree of correlation between two quantitative methods across the assay range may still be low. In all cases, any alternative in vitro strategy must be shown to be fit for purpose and should provide the same, or greater, level of assurance that the CQA in question is adequately controlled and that product safety and efficacy remain consistent with the lots evaluated in clinical trials. Once an in vitro alternative has been shown to be fit for purpose and approved by the NRA, there is no scientific justification for reverting to a former in vivo test, including, for example, when assessing the impact of process changes in comparability exercises.

In addition to the replacement of animal-based quality control methods with in vitro alternatives, consideration should also be given to the complete removal of an in vivo test from a quality control scheme where scientifically justified. In some cases, removing an animal test previously used for quality control purposes can be based on the assurances provided through the implementation of GMP, use of validated manufacturing processes and appropriate quality control measures during manufacture. Alternatively, the information and assurance obtained from a particular in vivo test may already be provided by tests or controls performed elsewhere in a validated production process. For example, some regulatory authorities have removed the requirement for a specific toxicity test on the final bulk and/or filled product for some toxoid vaccines in cases where the safety of the product has been assured by a sensitive specific toxicity test performed earlier in the process *(35)*. The removal of an animal test may also be proactively recommended following scientific demonstration and consensus that the test itself is uninformative and not fit for purpose, as was the case, for example, with the WHO-recommended discontinuation of the innocuity test *(14, 15)*.

While the focus of these WHO Guidelines is on the replacement or removal of animal tests for the quality control of biological products, it is acknowledged that until this can be fully implemented, the use of animals will continue for some products. During this transitional phase, high standards of animal housing, husbandry and care, in accordance with local and international regulatory frameworks, should be applied to minimize animal suffering. Pain, suffering and distress in experimental animals are all strongly associated with increased variability in experimental data, leading to reduced scientific reliability and reproducibility. Such data variability reduces statistical power, thus necessitating larger sample sizes and additional studies to reach meaningful conclusions. Where animal testing cannot yet be replaced or removed, improving animal welfare will enhance data quality and reproducibility, improving both the scientific integrity and efficiency of quality control testing schemes *(36–43)*. It may also be possible to refine the experimental approach to cause less harm and suffering to laboratory animals through the use of alternative, more humane end-points, or to optimize the experimental design to reduce the number of animals needed. However, the use of reduction or refinement approaches, such as the use of serological assays in place of challenge models or of single-dilution assays, should no longer be the ultimate goal. Instead, existing methods involving these strategies should become targets for replacement or removal approaches as they retain the high variability of animal tests while potentially limiting the ability to monitor product consistency. Consideration should also be given to the use of reliance schemes among NRAs/NCLs *(44)* and to the sharing of test data where relevant, as this would also reduce the total number of animals required to release a product lot.

Although it is recognized that the complete replacement or removal of animal tests for the quality control of biological products will not be immediate, there is increasing awareness of the considerable scientific and other limitations and challenges associated with the continued use of such tests. In line with the guidance provided in the current document, the development, validation and implementation of fully in vitro alternative approaches to animal tests, or the removal of animal tests shown to be unfit for purpose and/or redundant due to the use of validated manufacturing processes and appropriate quality control, is now strongly and unequivocally recommended.

## 5. Adventitious agent testing

Adventitious agents are contaminating microorganisms of the cell culture or starting/raw materials that have been unintentionally introduced into the manufacturing process of a biological product *(45)*. It is vital that all materials of biological origin used in the manufacture of vaccines and other biological products are shown to be free of adventitious agents. There are currently several in vivo and in vitro assays and testing modalities used to ensure the absence of adventitious agents *(46)*. These assays are applied across all aspects of the manufacturing process, including starting materials, process intermediates and drug substances. Testing of raw materials can also be done where justified.

### 5.1 In vivo adventitious agent testing

In vivo adventitious agent testing is performed as part of the qualification of starting materials (such as cell substrates and virus seeds) used in the manufacturing process of biological products, and for testing the crude unpurified bulk harvest to ensure that no viruses or other contaminating microorganisms were unintentionally introduced during the manufacturing process. In vivo adventitious agent testing is performed by inoculating the test sample into suckling and adult mice, embryonated eggs and, in some cases, guinea-pigs and rabbits. The animals are then observed for clinical signs or pathologies associated with infection for a defined number of days *(46, 47)*.

In vivo adventitious agent tests used to qualify cell banks, particularly of rodent origin (and in some cases the subsequent manufacturing process) include antibody production tests *(46)*. These tests are performed in hamsters, rats and mice to detect specific rodent viruses. The test sample is inoculated into one or more species, and after a defined amount of time, the animals are tested for antibodies against specific potential adventitious viruses. An overlapping approach with regard to the species used may be taken to address sensitivity differences among rodent species to the specific viruses being tested for.

With the adoption of the ICH Q5A(R2) Harmonised Guideline in November 2023 *(48)* and the publication of the new general chapter entitled “High-throughput sequencing for the detection of viral extraneous agents (2.6.41)” in the European Pharmacopoeia in October 2025 *(49)*, there is broad international recognition that all in vivo adventitious agent assays and antibody production tests can be replaced with molecular methods such as polymerase chain reaction (PCR) and high-throughput sequencing (HTS) based on either targeted or non-targeted detection *(50, 51)*. The use of these alternative methods is recommended.

### 5.2 Test for haemadsorbing and haemagglutinating viruses

Many viruses express glycoproteins that are capable of binding red blood cells, which in turn can result in haemadsorption and haemagglutination. A test for viruses that can cause haemadsorption and/or haemagglutination is performed as part of the in vitro cell-based test for adventitious agents. The presence of these adventitious agents has commonly been tested for using red blood cells from one or more sources (including human, guinea-pig, chicken, and

in some cases non-human primate) as the interaction between the cells and any viruses present may vary. However, given the low sensitivity and specificity of the test, its replacement with alternative approaches, such as molecular methods, is recommended *(48, 49)*.

## 5.3 Test for mycobacteria

Historically, guinea-pigs have served as a model for disease caused by several mycobacterial species. Based on this model, an in vivo test was developed that has previously been used to test for the presence of virulent mycobacteria in biological products *(52)*. Despite the subsequent replacement of the guinea-pig test with a validated culture-based test in the European Pharmacopoeia *(53)*, and broad acceptance of the culture-based test, the guinea-pig test is still detailed in several guidelines, including previously published WHO Recommendations and Guidelines. However, the use of the culture-based assay or PCR-based approaches instead of the guinea-pig test is now recommended. In addition, HTS approaches are also being developed to detect the presence of mycobacteria that may contaminate the manufacturing process of vaccines and other biological products *(54)*. Molecular methods have the advantage of being able to detect all mycobacteria (not just virulent strains), making such approaches a significant improvement over the guinea-pig test.

## 5.4 Test for avian viruses

Inactivated rabies vaccines, live attenuated yellow fever vaccines and live attenuated influenza vaccines, and their associated virus seed banks, are manufactured in embryonated eggs or in primary chicken fibroblasts derived from embryos. It is therefore essential to confirm the absence of adventitious avian agents in the virus seeds, control eggs/cell substrate and crude harvest/drug substance.

The current WHO Recommendations documents on inactivated rabies vaccines, live attenuated yellow fever vaccines and live attenuated influenza vaccines *(55–57)* list testing for avian viruses in embryonated eggs as part of adventitious agent testing requirements. However, it is recognized that HTS or virus-specific PCR assays are viable replacements for this assay and their use is strongly recommended.

## 5.5 Conclusions

The risk of adventitious agents is product specific and, where possible, can be reduced by using chemically defined animal-free media during production. Manufacturers should conduct a thorough risk assessment and develop a robust strategy for the control of adventitious agents. It is recommended that all adventitious agent testing performed in animals or using materials sourced from animals on a routine basis be replaced with suitable culture-based tests or molecular methods (PCR or HTS). Molecular methods can also supplement or replace the in vitro cell culture assays, and can be particularly useful where the viral vector or viral vector-derived product cannot be neutralized, resulting in assay interference, or where there is the possibility of cell toxicity due to the nature of the test material.

Despite the challenges associated with the design and validation of molecular methods (particularly HTS), such methods are increasingly recognized as having important advantages, including higher sensitivity and selectivity, and increased breadth of detection (especially when using a non-targeted approach) *(46, 49, 52, 58, 59)*. Positive results, if obtained, may not necessarily indicate the presence of infectious agents, and in such cases, prompt follow-up investigations should be conducted *(55, 60)*. Given the considerable differences in technologies and their associated characteristics, head-to-head comparisons intended to establish a correlation between in vivo methods and in vitro alternatives are not required. Additional considerations around the design, validation and implementation of molecular methods are discussed in relevant guidelines *(30, 49, 50)*.

## 6. Pyrogenicity and endotoxin testing

Pyrogens are substances capable of inducing a rise in body temperature (fever) when injected into humans or other animals through the activation of the innate immune system. Pyrogens may originate from microbial sources (for example, bacteria, fungi and viruses) or non-microbial sources (for example, rubber particles, microscopic plastic particles or metal compounds in elastomers). Microbial pyrogens can be further classified into two types: endotoxins (lipopolysaccharides shed from gram-negative bacteria) and non-endotoxin pyrogens (such as lipoproteins, peptidoglycan, lipoteichoic acid, double-stranded RNA, single-stranded RNA and CpG motifs).

Due to their potentially very serious health risks, the sensitive and accurate detection of pyrogens, and demonstration of their removal to levels generally accepted to be safe, are key requirements in the development of parenteral drugs *(61–63)*. Pyrogen levels should therefore be monitored and controlled throughout the manufacturing process.

Several test methods exist for the detection and/or quantification of pyrogens. Such tests can be classified based on the type of pyrogen they detect and on the need for animal materials to perform the test. Historically, the rabbit pyrogen test (RPT) was the only possibility until the development of the *Limulus* amoebocyte lysate (LAL) and *Tachypleus* amoebocyte lysate (TAL) assays. Based on amoebocytes obtained from horseshoe crabs, these assays were widely used in the 1980s to detect endotoxins. Since that time, there has been an exponential increase in the use of pyrogenicity assays, partly due to the rapid development of newer drugs and devices. More recent assays developed for pyrogenicity/endotoxin testing include the monocyte activation test (MAT) for the detection of all pyrogens and the recombinant Factor C (rFC)/recombinant Cascade Reagent (rCR) assays for the detection of bacterial endotoxins. These in vitro assays are superior or equivalent to the traditional animal-based test methods and are recognized and implemented in several pharmacopoeias, including both the European Pharmacopoeia and U.S. Pharmacopeia. As a result, the MAT and rFC/rCR assays offer excellent opportunities for the replacement of the RPT and LAL/TAL assays respectively.

### 6.1 Pyrogenicity testing

The RPT was developed more than 100 years ago, when manufacturing processes and environments were poorly controlled compared to current standards, and products typically contained high levels of pyrogens. The test involves the intravenous administration of test materials into rabbits and then measuring any rise in body temperature. Testing methods are described in different pharmacopoeias, which vary slightly in detail but follow the same basic principles.

Although the RPT is able to detect both endotoxins and non-endotoxin pyrogens *(64, 65)*, it also has several significant limitations. The assay is qualitative in nature and, as a result, is unsuitable for products that are inherently pyrogenic and for which an accurate determination of pyrogen levels may be of benefit *(64–67)*. In addition, it has a high degree of variability as its results can be influenced by the stress levels of the rabbits used, their prior exposure to pyrogens and the inherent variability of animal assays. Furthermore, due to its limited sensitivity, the presence of low levels of endotoxins and non-endotoxin pyrogens may not be detected *(63, 68)*. Rabbits can also develop tolerance following repeated exposure to some pyrogens. Consequently, a negative RPT result does not necessarily confirm the absence of pyrogens.

Recognition of the limitations of the RPT led to the development of the MAT in the late 1990s, with various versions of the assay becoming commercially available shortly afterwards. MATs that use anticoagulated whole blood, human peripheral blood mononuclear cells (PBMC) or monocytic cell lines are available, some as ready-to-use kits *(62, 63, 69)*. All versions of the MAT are based on the detection and/or quantification of the activation of transcription factors or the release of pro-inflammatory cytokines by human monocytes in response to endotoxins and non-endotoxin pyrogens. The readout is generally obtained through quantification of the mediator by an immunobinding method such as enzyme-linked immunosorbent assay (ELISA).

MATs require the use of qualified blood, PBMC or cell lines, and should be adequately validated to ensure robust performance over the product life-cycle. Detailed information on conducting the MAT, and important considerations when performing the test on vaccines containing inherently pyrogenic components, are provided in specific European Pharmacopoeia texts *(70, 71)*.

MATs based on the use of human blood or PBMC (individual or pooled) closely mimic human immune responses and, given their sensitivity, can offer advantages when assessing pyrogenicity in nonclinical studies. However, human blood and PBMC may be more susceptible to handling artefacts and may require frequent qualification due to donor variability. MATs that use monocytic cell lines may therefore be easier to reproduce and standardize. As a result, there are potential benefits to using MATs based on monocytic cell lines for routine quality control testing (over the product life-cycle), especially when consistent manufacturing has been demonstrated. MATs in ready-to-use formats based on frozen PBMC or monocytic cell lines are commercially available, and engineered cell lines continue to be developed *(72)* thus potentially improving access to such assays.

Compared to the RPT, the MAT has several advantages, including its ability to more closely reflect human immune responses. MATs also show improved reliability and reproducibility, and have high sensitivity to low levels of endotoxins and non-endotoxin pyrogens. Furthermore, MATs can be quantitative when used in conjunction with appropriate

reference standards and controls *(62, 63)*. This makes them suitable for testing products that are inherently pyrogenic and for which an accurate assessment of pyrogen level is required.

Given the improvements in assay performance and the increasing accessibility of MATs, the replacement of the RPT with the MAT is strongly recommended *(73)*. The European Pharmacopoeia General Chapter 5.1.13 on pyrogenicity provides guidance on the selection and implementation of a suitable test for pyrogenicity (bacterial endotoxin test or MAT) based on risk assessments *(74)*. It should be noted that the MAT is a relative assay, and that a prerequisite for such assays is statistical similarity in the form of comparable dose–response curves for both the reference and test materials (that is, like against like). This can be achieved by using a product-specific in-house reference material (for example, a representative drug product lot) *(75)*.

## 6.2 Endotoxin testing

The LAL and TAL assays were initially developed as alternatives to the RPT. These assays are enzyme-based tests in which horseshoe crab amoebocytes form a gel clot in the presence of endotoxins. Information on conducting these assays, and additional guidance, is provided in several pharmacopoeias.

Although LAL/TAL assays cannot detect non-endotoxin pyrogens, they demonstrate a high sensitivity for endotoxins, particularly when using kinetic methods, and can be at least 100 times more sensitive to endotoxin compared to the RPT *(61)*. By including appropriate controls and recognized international reference standards, endotoxin levels can also be accurately quantified using LAL/TAL assays. Thus, the assay is suitable for products for which robust quantification of residual endotoxin is necessary. It is also suitable for use as an in-process control to monitor microbial control throughout manufacturing (including as part of environmental monitoring, water control, cleaning validation, etc.). A further advantage lies in its ease of use, with the presence of endotoxin easily detected and quantified.

However, LAL/TAL assays also have several limitations. Amoebocyte lysate is a heterogeneous aqueous extract of horseshoe crab blood, and as such contains several proteins in addition to the endotoxin sensor (Factor C). These proteins can interact with the components of drug products and influence the assay readout. One such example is the Factor G protein pathway, which can be activated through the detection of β-glucans and cellulosic residues, leading to signal enhancement or false positives. Due to their dependence on a limited natural resource, LAL/TAL assays may also be susceptible to supply chain shortages and cost fluctuations *(76)*. These and other concerns around animal welfare and assay sustainability have led to the development of alternative approaches based on the use of recombinant horseshoe crab proteins *(77, 78)*.

The first of these alternatives – the rFC assay – uses a recombinant version of Factor C instead of horseshoe crab blood, along with a fluorogenic peptide substrate *(79, 80)* and became commercially available in the early 2000s. Due to the absence of Factor G, the rFC assay shows a higher specificity for endotoxins compared to LAL/TAL assays *(68, 76)* and is also reported to be at least as sensitive *(81)*.

A second alternative – the rCR assay – became commercially available in 2021. This assay uses recombinant versions of the three proteins involved in the LAL/TAL clotting cascade (Factor C, Factor B and the pro-clotting enzyme), along with a chromogenic substrate. By excluding the Factor G protein, it retains several of the advantages of the rFC assay *(68, 76)*. The rCR assay is now included in the U.S. Pharmacopeia *(71)* and efforts are under way to facilitate its wider adoption.

Studies conducted to date have demonstrated the comparability (and potential superiority) of assays based on the use of recombinant versions of horseshoe crab proteins to conventional endotoxin assays *(68, 78, 81)*. As a result, the replacement of LAL/TAL assays (all of which use animal-sourced reagents) with rFC/rCR assays is strongly encouraged. Both of these recombinant protein assays are recognized as viable alternatives to LAL/TAL assays.

## 6.3 Conclusions

Given the ubiquitous nature of pyrogens and their numerous potential sources (raw materials, personnel, equipment, container closures, etc.), a holistic approach to their control during manufacture is recommended. Manufacturers should consider using a risk-based approach to identify all relevant pyrogens that quality control testing should cover. A tiered approach may be used in which all pyrogens are checked for during product development and then only those potentially present are tested for during routine manufacture. The European Pharmacopoeia General Chapter 5.1.13 on pyrogenicity provides guidance on the selection and implementation of a suitable test for pyrogenicity (bacterial endotoxin test or MAT) based on risk assessments *(74)*.

Risk assessments should take into account the nature of the product, the starting/raw materials and the product-related impurities. Careful consideration should be given to the selection and implementation of pyrogenicity/endotoxin assays at the appropriate stages of product development, manufacture and quality control. In all cases, the use of the RPT is not recommended and where currently used, it should be replaced with the alternative pyrogenicity/endotoxin tests described above. Where there is a risk of non-endotoxin pyrogens being present, the use of the MAT is recommended. In cases where non-endotoxin pyrogens are unlikely to be present, endotoxin testing using the rFC assay or rCR assay is recommended.

# 7. Neurovirulence testing

Live attenuated viral vaccines use infectious viruses with reduced virulence in humans to induce protective immunity and have been used successfully to control diseases since the 1930s. The virus strains used to prepare live vaccines are often attenuated from wild-type viruses that are associated with severe neurological disease, such as polio, measles and mumps. Additionally, vaccine production strains that are attenuated through serial passage in tissues of the central nervous system (CNS) may acquire neurovirulence properties, as was the case with the French neurotropic vaccine (FNV) passaged in mouse brains. Due to the severity and often irreversible nature of neurological damage caused by viral infection, the testing of live attenuated viral vaccines for neurovirulence has long been a regulatory requirement for product

development and commercial production. Ever since the introduction of the monkey neurovirulence test (MNVT) for the control of virus seed lots of yellow fever vaccine in 1945, neurovirulence testing has played an important role in ensuring the safety of live attenuated viral vaccines.

Historically, the MNVT has been used for both the nonclinical assessment of novel viral vaccines and for the quality control of several licensed live viral vaccines. However, the need to perform neurovirulence testing for some live viral vaccines has recently been subject to scrutiny *(82–84)*, due in part to increasing understanding of the mechanisms and genetic basis of attenuation. The following sections outline the history, scientific basis and limitations of animal-based neurovirulence testing for several live attenuated vaccine products.

## 7.1 Yellow fever vaccine

Wild-type yellow fever virus strains are predominantly viscerotropic in primates, including humans *(85)*. However, it has been shown that such viruses can be both viscerotropic and neurotropic *(86)*. In the 1930s, virulence studies were conducted in monkeys to aid the development of two live attenuated yellow fever vaccines – namely, FNV and the 17D vaccine *(87)*. FNV contained a yellow fever virus strain that had been passaged in mouse brain more than 100 times and shown to have increased neurovirulence but decreased viscerotropism in monkeys *(88)*. The use of FNV was discontinued in the 1960s due to a high incidence of encephalitic reactions in children following vaccination. The 17D vaccine was derived from wild-type yellow fever virus passaged in tissue cultures prepared from embryonated chicken eggs, which resulted in the loss of viscerotropism in both monkeys and humans, along with reduced neurovirulence in monkeys and mice *(89)*. All currently licensed live attenuated yellow fever vaccines are produced using three substrains derived from the 17D virus strain. While the precise molecular determinants of attenuation and virulence have not been established, the 17D vaccine has been shown to be genetically stable, which likely contributes to its safety *(90)*.

In 1941, cases of encephalitis (including one fatality) as well as a higher incidence of severe systemic reactions (some including CNS signs) were reported in Brazil in individuals who had received different lots of yellow fever vaccine prepared from the same 17D-derived substrain (NY17D-104) *(91)*. Studies in monkeys using intracerebral inoculation showed that the ability to produce encephalitis varied among the different 17D-derived substrains, with NY17D-104 associated with the highest incidence *(92)*. The MNVT was subsequently introduced for the control of yellow fever virus seeds used for vaccine production. A seed lot system was also introduced for yellow fever vaccine production, which has since become a key manufacturing control strategy for all biological products. Such changes have greatly improved the lot-to-lot consistency of yellow fever vaccines. However, despite these and other improvements to manufacturing control implemented since the 1940s, rare adverse events of yellow fever vaccine-associated neurotropic disease (YEL-AND) and viscerotropic disease (YEL-AVD) continue to occur. Extensive investigations into these rare adverse events have not identified any quality issues with the vaccine (such as genome mutations) that may have been responsible *(93)*.

The requirement to perform the MNVT on secondary yellow fever virus seed lots was first established by the United Nations Relief and Rehabilitation Administration in 1945 and involved assessing neurovirulence based on clinical signs of encephalitis *(94)*. Additional changes have been introduced to the MNVT over the years, with current WHO Recommendations requiring that the neurovirulence of both the master and working virus seed lots be tested in monkeys based on the scores of both clinical and histological evaluations. The seed lot passes the test if the overall mean scores for monkeys inoculated with the seed virus are not significantly greater (at the 5% significance level) than the overall mean scores for monkeys inoculated with the reference virus *(95)*. There is currently no international reference standard for this test and vaccine manufacturers generally use a homologous preparation known to produce a satisfactory product as an in-house reference. The current WHO MNVT for yellow fever vaccine has been designed to also allow for semi-quantitative assessment of the test sample. However, the poor accuracy of clinical scoring in non-human primates, along with the use of in-house reference preparations with very low residual neurovirulence, may increase the risk of failing virus seed lots that are sufficiently attenuated for vaccine production, as has been reported by one vaccine manufacturer *(84)*. The MNVT performed for the control of yellow fever virus seed lots has clinical relevance as it can be used to identify virus preparations that may lead to a high incidence of encephalitis in human vaccine recipients. However, the test is based on an assumed correlation between the safety profile of vaccines in humans and parameters measured in monkeys. Under certain circumstances, mice may be used instead of monkeys to assess the neurovirulence of various attenuated yellow fever viruses (or of chimeric viruses based on attenuated yellow fever virus) for the quality control of virus seed lots.

The precise mechanism and genetic basis of neurovirulence of yellow fever viruses are poorly understood. Nevertheless, the level of residual neurovirulence of an attenuated virus is determined by the viral genome sequence. As a result, the whole genome sequence of new virus seed lots should be compared against those of historical seed lots used to manufacture vaccine lots demonstrated to be safe in clinical studies and/or routine use. The sequence of the virus seed lots should be determined using a validated molecular method. Sequence heterogeneities across the entire genome, including single nucleotide polymorphisms, are permitted if shown to have no impact on vaccine safety. This approach is recommended as a replacement for the current in vivo neurovirulence testing used for the quality control of yellow fever virus seed lots used for vaccine production.

## 7.2 Oral poliomyelitis vaccine

Wild-type poliovirus of all three distinct serotypes (types 1, 2 and 3) can enter the human CNS and replicate in motor neurons. The resulting destruction of these neurons can then lead to temporary or permanent paralysis. In the 1950s, Dr Albert Sabin discovered that all attenuated poliovirus strains retained varying degrees of neurotropism in monkeys and that this residual neurotropism could be measured quantitatively based on the incidence of paralysis *(96)*. The use of the MNVT during the clinical development of Sabin oral poliomyelitis vaccine (OPV) has demonstrated that vaccines containing attenuated poliovirus strains associated with low levels of neurotropism in monkeys are generally safe and efficacious in humans. However, it

was later discovered that all three types of Sabin OPV could revert to neurovirulent forms, causing poliomyelitis in rare cases *(96, 97)*. It is now well established that all three types of Sabin OPV are genetically unstable and that the neurovirulent form present in the final OPV product is linked to reversions in viral 5′-UTR, typically representing less than 0.1% of the virus population *(98–100)*. As a result, neurovirulence testing on virus seeds and monovalent bulks must be performed to ensure the safety of Sabin OPV. Recently, a rationally designed and more genetically stable strain of type 2 poliovirus has been developed. This novel OPV type 2 (nOPV2) strain exhibits minimal reversion to the virulent form in both animal models and humans *(101, 102)*.

The standardized MNVT procedure for OPV set out by WHO is based on intraspinal inoculation and subsequent assessment of neurovirulence based on histological examination. Lesions in the CNS are scored and a comparison made between the sample being tested and the WHO international standard. OPV bulk and seed lots pass if the lesions are not greater than those caused by the international standard. Although the MNVT has long been recognized as a key control test that has contributed to the good safety record of Sabin OPV, it also has a number of limitations, with its results not always predictive of the residual neurovirulence of attenuated polioviruses in humans. For example, in 1962, a new attenuated type 3 poliovirus passed the MNVT and showed a lower residual neurovirulence than the Sabin type 3 strain. However, the subsequent clinical study of the new strain led to an extensive outbreak of vaccine-related poliomyelitis *(103, 104)*. In addition, based on MNVT results, one review of data on 80 Sabin type 3 OPV lots manufactured between 1964 and 1983 in the USA indicated that type 3 OPV was less neurovirulent than type 1 OPV. However, real-world evidence indicates that type 3 OPV is more frequently associated with vaccine-related poliomyelitis than type 1 OPV. Such experiences have highlighted that monkey neurovirulence testing alone is insufficient to guarantee the safety of OPV.

The transgenic mouse neurovirulence test (TgmNVT) was developed in the 1990s and uses mice expressing the human gene encoding the cellular receptor for poliovirus (CD155). Following a WHO-led collaborative study, the TgmNVT was recommended by WHO as an alternative to the MNVT for all three OPV types *(105)*. Mouse neurovirulence testing is based on clinical scoring to allow for determination of the number of normal and paralysed transgenic mice following intraspinal inoculation. Neurovirulence testing (in either monkeys or transgenic mice) has long been a key requirement for monitoring the safety and consistency of OPV production by ensuring that the neurovirulence of commercial lots is controlled at no more than that of the international standard. However, in addition to the recognized shortcomings of the MNVT noted above, the outcomes of such neurovirulence testing may also vary depending on the choice of experimental animal (monkeys or transgenic mice) and route of injection (intraspinal or intracerebral) *(105, 106)*.

Advances in molecular biology in the 1990s led to the identification of the principal nucleotides responsible for the attenuation of all three types of Sabin OPV and to the subsequent development of the mutant analysis by PCR and restriction enzyme cleavage (MAPREC) assay *(99)*. This molecular method is used to quantify the percentage of reversion for one or two of the important nucleotides responsible for attenuation in each of the three Sabin strains. While MAPREC can be used to screen bulks and to avoid the need for animal neurovirulence tests on clearly positive samples, it cannot completely replace the MNVT or

TgmNVT as other nucleotides also contribute to the attenuated phenotype. Despite being a WHO-recommended method for quantifying mutations in poliomyelitis vaccines, MAPREC is able to detect only a small number of mutations. In addition, the method is technically demanding and the maintenance of competency difficult.

As an alternative to both in vivo testing and MAPREC, approaches based on the use of HTS are scientifically far more robust and technically less challenging. HTS offers the potential of whole-genome analysis for routine quality control once manufacturing consistency has been established, and is capable of measuring the level of polymorphisms at each genome nucleotide with a detection sensitivity as low as 1% *(100, 107)*. WHO recommends that a validated whole genome HTS assay be used to replace the in vivo neurovirulence test for the routine manufacturing control of all OPVs (Sabin and nOPVs) and for seed virus testing for inactivated poliomyelitis vaccine products *(108–110)*.

## 7.3 Mumps vaccine

Mumps virus is a major cause of aseptic meningitis in unvaccinated populations. Because of the high neurotropism of wild-type mumps viruses, neurovirulence testing of candidate live attenuated mumps vaccines is generally required by NRAs. Historically, such testing has been performed in monkeys based on an evaluation of mumps virus specific neuropathology, mainly periventricular inflammation and neuronal necrosis, following intracerebral inoculation. However, two independent studies conducted in 1999 indicated that the results of neurovirulence testing in monkeys do not correlate with the risk of mumps vaccine related neurovirulence in humans *(111, 112)*. It is therefore not surprising that such testing had failed to detect residual neurovirulence in an attenuated mumps virus strain (Urabe Am9) previously used by several manufacturers to produce live attenuated mumps vaccines. Several aseptic meningitis cases associated with live vaccines derived from Urabe Am9 were reported in Canada, Japan and Europe in the late 1980s. A more sensitive test using neonatal rats that can differentiate attenuated strains with varying levels of residual neurovirulence has been developed *(112, 113)*. Nevertheless, the development, validation and implementation of alternative in vitro tests (for example, based on evaluating viral genome sequence consistency) is recommended.

## 7.4 Other viral vaccines

As with attenuated mumps viruses, monkey neurovirulence testing is also not suitable for the control of residual neurovirulence of live attenuated measles, rubella and varicella-zoster viruses *(82, 83)* as the neuropathological manifestations of these viruses in monkeys do not correlate with their known neurovirulence in humans. As a result, neurovirulence testing in monkeys does not account for the many potential mechanisms of neurovirulence in humans. Furthermore, the relevance of testing smallpox vaccine for neurovirulence in monkeys is also questionable given the lack of viraemia. WHO acknowledges the need for suitable alternatives and strongly encourages the development of non-animal-based approaches for evaluating the residual neurovirulence of viral vaccines.

## 7.5 Conclusions

At the nonclinical stage, the potential neurovirulence of a new attenuated virus strain should be evaluated based on all available scientific data and information. Neurovirulence testing in a suitable animal model should be considered in cases where the wild-type virus is neurovirulent or was passaged through tissues of the CNS – or where a chimeric virus has components that may be neurovirulent.

It should be noted that all neurovirulence tests in experimental animals (such as monkeys, mice and rats) were established based on an assumed correlation between the neuropathological manifestations of the attenuated virus strain in animals and the safety profile of the same strain in humans established through clinical studies. However, due to physiological differences between experimental animals (including non-human primates) and humans, it is not possible to predict for certain the residual neurovirulence of a live attenuated vaccine in humans based only on neuropathological manifestations in animal models. In addition, in vivo neurovirulence testing alone cannot eliminate the possibility of the rare adverse neurological reactions associated with yellow fever vaccines and OPV, which are considered to be intrinsic to such vaccines. It is anticipated that emerging technologies will lead to the development of suitable in vitro models which will replace in vivo nonclinical neurovirulence testing.

Each virus seed and all vaccine lots used in clinical studies should be characterized by full-length genome sequencing, in part to facilitate the subsequent use of molecular methods for quality control of the commercial product at appropriate manufacturing steps (for example, virus seed lots). If the virus strains used for commercial production of live attenuated vaccines are shown to be genetically unstable (for example, Sabin OPV), control of residual neurovirulence is generally required for virus seeds and for each commercial lot, using appropriate molecular methods based on the consistency approach. The requirement to perform in vivo neurovirulence tests specified historically in previously published WHO Recommendations, Guidelines and other guidance documents can now be replaced with a requirement to demonstrate whole viral genome sequence consistency between commercial virus lots and the virus preparation shown to be safe and efficacious in clinical studies. Advances in molecular biology have led to a greatly improved understanding of the mechanisms and genetic basis of attenuation (and of reversion to neurovirulence), which in turn will lead to the development of ever more reliable methods, such as HTS, for the control of residual neurovirulence in biological products.

# 8. Potency testing

The potency of biological products has traditionally been measured using in vivo relative potency assays in which the response of the test sample is compared against a reference standard of known activity. Typically for vaccines, the formulated product is introduced into an animal model (such as mouse, guinea-pig or rat) in a defined test format. The test may involve subsequent challenge of the animals with the relevant pathogen or toxin to determine

the level of protection afforded by the product by watching for signs of disease or death. Alternatively, sera may be collected from immunized and unchallenged animals and then tested for the presence and quantity of relevant antibodies.

When transitioning from in vivo to in vitro potency testing, it is important to understand the CQAs of the product and its mode of action to determine which product characteristics are most representative of clinical efficacy. It is then essential to be able to demonstrate the consistent production of lots shown to have similar characteristics to lots that were demonstrated to be safe and efficacious in clinical studies or routine clinical use. In this context, the superior reproducibility and repeatability of well-designed in vitro tests compared to in vivo tests significantly increases their utility when comparing the results obtained for a test lot to those obtained for a lot demonstrated to be safe and efficacious in pivotal clinical studies *(114, 115)*. Such a consistency-centred approach implies both a well-characterized and controlled production process and an integrated quality control scheme that results in a product with a well-established safety and efficacy profile *(114, 115)*.

The established concept of "one-to-one" (also known as "head-to-head") comparison for method replacement, whereby a statistical correlation is demonstrated between the results obtained using the different assays, may not be of value when transitioning to in vitro methods due to the inherent variability and historical validation status of the in vivo methods. In addition, in vivo and in vitro methods typically assess potency differently and produce different readouts that are not comparable. Furthermore, in vivo tests measure a complex response (including the adjuvant effect in the case of vaccines), and with a high degree of variability. Such tests provide little insight into the differences in quality behind any observed changes in potency between lots. By contrast, in vitro tests typically measure specific CQAs, and with higher precision. Therefore, when suitably combined, such in vitro tests can be used to more precisely determine the final quality of the product. The European Pharmacopoeia has recognized this and provides a general chapter to guide users during in vivo replacement exercises *(30)* while others are developing similar principles *(114, 115)*. In summary, an inability to assess or demonstrate correlation with an in vivo assay does not inherently mean that an in vitro assay will be unsuitable, and so the focus of assay evaluation should instead be placed on assessing its suitability for the intended purpose.

Despite the challenges, different approaches can be used to assess the suitability of in vitro potency tests. The goal is to have sufficient data demonstrating that the in vitro assay(s) are capable of measuring CQAs that have been scientifically justified to be relevant both for efficacy and for ensuring that the product quality profile stays within the desired range. Any changes in quality should be assessed in comparison to the expectations defined for the same CQAs of lots shown to be safe and efficacious during clinical studies or routine use.

When designing an approach to assess product potency, both content and functionality must be considered. In some cases, a single assay can be used to measure both. One example for vaccines would be an immunochemical assay using a well-characterized monoclonal antibody (or antibodies) against a vaccine epitope known to be a target for neutralizing antibodies (for example, when assessing the D antigen potency of inactivated poliomyelitis vaccines) *(116)*. The identification and characterization of monoclonal antibodies suitable for the desired purpose is a critical step in assay development, as was illustrated during the development of in vitro assays for rabies, diphtheria, tetanus and acellular pertussis vaccines

*(117–119)*. Such assays would ideally target an epitope or epitopes that are conformational so that they also have stability-indicating capacity *(30)*. Cell-based assays provide another example where a single assay may reflect both content and functionality, provided they are quantitative and have low variability *(120, 121)*. If a single assay does not provide adequate information, then more than one assay may be needed. An example of this approach is provided by messenger RNA (mRNA) vaccines where quantitative tests for RNA content are combined with other tests used to assess the CQAs related to potency (for example, mRNA purity/integrity and encapsulation). Sequence confirmation and semi-quantitative cell-based expression assays can also be used during mRNA vaccine evaluation to provide a complete picture *(122, 123)*. The integrated overall control strategy may include tests performed at different stages of production. For example, vaccine antigen purity may be evaluated at the pre-adsorption stage, with antigen content and percent adsorption evaluated at the final container stage.

The potency testing of human recombinant erythropoietin (EPO) – a product which is dosed in IU – provides another example of where in vitro tests can be introduced for routine batch release, consistency monitoring and other quality control purposes. It may be scientifically possible to establish a combination of in vitro (cell-based) assays (to assess EPO receptor binding) and physicochemical tests to assess quality attributes critical for biological activity (such as assessment of sialic acid content). This combination of analytical approaches could then be used to predict the biological activity of the EPO product lots in vivo. However, the use of such an approach would require demonstration of its ability to detect out-of-specification lots. The approach would also depend on an in-depth statistical analysis of historical (that is, animal) potency data generated using a significant number of in vivo assays, across a large number of lots of EPO product, to support the justification.

More straightforwardly, an in vitro method, based on proliferation of an EPO-responsive cell line, may be used to measure the potency of EPO preparations relative to an in-house standard of identical origin, the potency of which (in IU) has previously been assigned using an in vivo assay *(124)*. This approach would allow for a significant reduction in the use of animals as the in vivo assay would only be required for the initial calibration of the in-house standard of identical origin. Subsequent assessment of the biological activity of product lots for quality control purposes would use the in-house standard in the in vitro assay. This approach is based on the fact that the biological activity of EPO exhibits a complex relationship between structure and function, and differences in the glycosylation profile of EPO may result in differences in the in vivo and in vitro bioactivities of EPO from different sources.

In all cases, the in vitro tests used should be able to discern any meaningful changes in product quality that will impact potency. Production lots shown by in vivo testing to possess distinct and reliably different activity levels (in particular, out-of-specification lots) are ideal test samples during the development of potential replacement in vitro assays. However, lots from regular production that do not meet the potency specifications are usually rare and the variability of in vivo assays makes it difficult to detect sufficiently distinct levels of potency over a tight enough range to monitor lot consistency. As an alternative, the capability of the in vitro assay may initially be assessed using samples of increasing/decreasing concentration generated by dilution of the active substance, ideally within a constant formulation matrix.

When replacing an existing in vivo assay for a legacy product, a statistically meaningful number of commercial lots tested with the in vivo assay should be evaluated using the new in vitro test to establish a baseline for specification setting. If available, borderline pass/fail lots, artificially altered lots or specifically manufactured lots are particularly useful in identifying relevant specification limits. For new products, in vitro tests for CQAs should be integrated as early as possible into product development and should be used to assess the CQAs of clinical lots and subsequent qualification/validation lots to facilitate the defining of suitable specifications. Samples that have been artificially altered through temperature, physical or chemical stress (for example, oxidation or pH change) can be very useful when assessing the stability-indicating potential of an in vitro method.

New opportunities continue to emerge for making the transition from in vivo to in vitro potency testing of biological products. For example, an in vitro assay for rabies vaccine based on an ELISA method using well-characterized antibodies that recognize the trimeric form of the glycoprotein is at an advanced stage in the European Directorate for the Quality of Medicines & HealthCare Biological Standardisation Programme *(117, 125)*. Efforts are also under way to develop in vitro assays for diphtheria, tetanus and acellular pertussis vaccines stemming from the activities of the VAC2VAC consortium *(4, 118, 119, 125–129)*. Encouragingly, developers of new products (including vaccines) are increasingly integrating the concept of in vitro method development into the nonclinical and clinical trial stages. While in vivo assays may currently still be used during early nonclinical product development, good product characterization and test design can avoid any requirement for in vivo quality control testing during subsequent commercial production.

## 8.1 Conclusions

The use of scientifically relevant control strategies for potency based entirely on the use of in vitro methods leads to better control of product consistency and thus to good quality medicines of assured safety and efficacy. A number of WHO Recommendations, Guidelines and other guidance documents provide examples of successful potency testing strategies that have never required the use of in vivo methods. For example, in the WHO Recommendations to assure the quality, safety and efficacy of pneumococcal conjugate vaccines, no in vivo tests are referenced for quality control *(130)*. However, in other such WHO documents, outdated recommendations for in vivo testing persist – for example, in the WHO Recommendations on poliomyelitis vaccines (inactivated) and WHO Recommendations on recombinant hepatitis B vaccines *(131, 132)* – despite the availability of established in vitro methods. Rather than awaiting the updating of previously published WHO Recommendations, Guidelines and other guidance documents in this regard, it is instead strongly urged that progress is now made towards the implementation of entirely non-animal-based approaches to potency testing.

The product-specific implementation of existing in vitro methods, and the development of new in vitro methods for potency assessment where gaps remain, are strongly encouraged. For example, the WHO Guidelines for the production, control and regulation of snake antivenom immunoglobulins *(133)* has sections that specifically consider the ethical use of animals, their use in quality control schemes and the development of alternative assays. Action

should be taken by manufacturers of medicinal biological products to drive such a shift through technical developments, ideally supported by consortium or common studies with multi-stakeholder engagement, such as VAC2VAC *(20)* and through NRA/NCL initiatives where relevant. These and other steps should be taken in full consultation with the NRA. International regulatory cooperation and convergence are also encouraged to accelerate the global acceptance of scientifically justified in vitro approaches to biological product potency assessment.

# 9. Specific toxicity

## 9.1 Diphtheria, tetanus and acellular pertussis vaccines

Specific toxicity testing is an essential part of routine lot testing for vaccines produced by the chemical detoxification of bacterial toxins (such as diphtheria, tetanus and pertussis toxins) to produce inactivated toxins (toxoids). Such testing is performed to provide assurance that the detoxification of these toxic starting materials is complete and irreversible. Historically, this testing has been performed using guinea-pigs, rabbits or mice (sensitized with histamine in the case of pertussis toxoid) and has been performed at different stages of the production process. Any non-animal alternative test for specific toxicity and reversion testing of these vaccine components needs to be specific and at least as sensitive as the existing animal model, such that assurance regarding the safety of these vaccine components is maintained. An in vitro approach to specific toxicity testing may be based on the use of a toxin-sensitive cell line or on the use of one or more assays that specifically target parameter(s) known to be essential for toxicity in vivo. Considerations and recommendations relating to specific toxicity testing for diphtheria, tetanus and acellular pertussis vaccines are provided in the following sections.

### 9.1.1 Diphtheria vaccine

A specific and highly sensitive non-animal method (Vero cell assay) is available for the detection of diphtheria toxin *(134)* and a method for performing this assay is described in the WHO Manual for quality control of diphtheria, tetanus and pertussis vaccines *(135)*. The current WHO Recommendations to assure the quality, safety and efficacy of diphtheria vaccines (adsorbed) refers to the Vero cell assay as an alternative to the animal model for testing bulk purified diphtheria toxoid, for both absence of toxin and non-reversion to toxicity *(136)*. Once validated, the Vero cell assay should be used for routine lot testing of bulk purified toxoid. The current WHO Recommendations also include a test for specific toxicity on the final bulk vaccine. However, provided the necessary assurance regarding detoxification of the toxin has been obtained from testing performed on the bulk purified toxoid, the specific toxicity test performed on the final bulk is redundant and can be omitted. With the introduction of the Vero cell assay at the purified bulk stage to replace the in vivo test, and removal of redundant toxicity testing at the final bulk and final lot stages, WHO recommends that in vivo testing for the toxicity of diphtheria vaccines should no longer be required, as is now the case in the European Pharmacopoeia *(137)*.

### 9.1.2 Tetanus vaccine

The current WHO Recommendations to assure the quality, safety and efficacy of tetanus vaccines (adsorbed) *(138)* makes reference to a guinea-pig assay used to confirm the absence of tetanus toxin in the bulk purified toxoid and the irreversibility of the toxoid. Reference is then made to a specific guinea-pig toxicity test on the final bulk vaccine. However, as with the corresponding WHO Recommendations for diphtheria vaccines outlined above, specific toxicity testing of the final bulk is now considered to be redundant, and can be omitted provided that the necessary assurances regarding detoxification have been obtained from the testing performed on the bulk purified toxoid.

Efforts are under way to develop scientifically relevant non-animal alternatives for the detection of tetanus toxin that could be applied to the toxicity testing of bulk purified tetanus toxoid. One example is the binding and cleavage (BINACLE) assay which takes into account the receptor binding and proteolytic activity of the toxin *(139)*. This assay has undergone extensive evaluation by the European Directorate for the Quality of Medicines & HealthCare *(140, 141)*, with results to date indicating that the assay is both sensitive and precise. The applicability of the method for testing different tetanus toxoids will need to be established on a case-by-case basis. Other alternative assays (such as cell-based assays) may also be suitable if sufficiently validated and shown to have adequate sensitivity. Where possible, data already obtained on the sensitivity of the guinea-pig assay to tetanus toxin should be used for such assessments rather than being specifically generated for this purpose, as this will avoid the need for additional animal studies. In general, any in vitro test should provide the same level of assurance as the guinea-pig assay with respect to acceptance or rejection of a lot.

### 9.1.3 Acellular pertussis vaccine

The scientifically relevant, specific and sensitive Chinese hamster ovary (CHO) cell clustering assay can be used to detect pertussis toxin *(142)*. Standardized protocols have been developed, including an indirect version of the assay for testing final bulk vaccine in the presence of adjuvant *(143, 144)*. The assay takes into account the receptor binding, translocation and enzymatic activity of pertussis toxin.

The WHO Recommendations to assure the quality, safety and efficacy of acellular pertussis vaccines *(145)* includes the now outdated recommendation to use either the in vivo mouse histamine sensitization test (HIST) or the CHO cell clustering assay to detect residual pertussis toxin activity in the non-adjuvanted pertussis toxoid. However, given the availability of the suitable and well-standardized CHO cell clustering assay, and the high inherent variability and animal welfare implications of the HIST, the in vivo test is no longer recommended for testing the residual activity of pertussis toxin in the purified toxoid *(146)*. In addition, the CHO cell clustering assay has been demonstrated to have greater sensitivity than the HIST *(147)* and lower variability when a standardized protocol is followed *(143)*.

Although a modified CHO cell clustering assay can be used to monitor residual pertussis toxin activity in the presence of an adjuvant *(144)*, detoxification should be controlled and verified using the CHO cell assay prior to adsorption. In line with the above guidance on diphtheria and tetanus vaccines, performing any specific toxicity test on the final bulk of acellular pertussis vaccines is also now redundant and can be omitted provided the necessary

assurance regarding detoxification has been obtained from testing performed on the bulk purified toxoid. Other non-animal-based approaches that may prove useful for the quality control of acellular pertussis vaccines have been developed and should be considered for use as part of the control strategy *(148)*.

### 9.1.4 Reversion to toxicity

For each of the above vaccine components (diphtheria, tetanus and acellular pertussis toxoid), current WHO recommendations include tests for reversion to toxicity in which samples are incubated at elevated temperature for 4–6 weeks prior to measurement of toxicity. However, a routine test for reversion is not required by all regulatory authorities. Manufacturers should validate the detoxification process to demonstrate that a stable toxoid is consistently produced that does not undergo reversion to toxicity during downstream processing, during storage under recommended conditions or during use. Once lack of reversion has been demonstrated to the satisfaction of the NRA/NCL, then routine reversion to toxicity testing should not be required. In addition, in the case of tetanus toxin, experimental evidence has indicated that the toxin loses activity when stored under the conditions used for the test for reversion to toxicity *(149)* – an observation that contributed to the decision to remove the requirement for a routine reversion test from one regional pharmacopoeia *(35)*. For pertussis toxoid, if a test for reversion is required, then the use of the HIST is no longer recommended. Even though a modified CHO cell clustering assay can potentially be used to monitor pertussis toxin activity in the presence of adjuvant, it is recommended that the assurance regarding the stable inactivation of pertussis toxin is provided by testing the non-adjuvanted toxoid, for which the standard CHO cell clustering method should be used.

## 9.2 Polysaccharide vaccines conjugated to a diphtheria or tetanus toxoid carrier protein

Current WHO recommendations for *Haemophilus influenzae* type b conjugate vaccines, meningococcal group A and C conjugate vaccines, pneumococcal conjugate vaccines and typhoid conjugate vaccines include a test for specific toxicity on the bulk conjugate (where a diphtheria toxoid or tetanus toxoid carrier protein is used). In each case, the control of specific toxicity should be performed for the carrier protein. Where available and appropriately validated, an in vitro assay should be used for this purpose – for example, the Vero cell assay for diphtheria toxoid. Where an in vitro assay is not yet available, the use of an in vivo assay at the control of the carrier protein stage (as opposed to the bulk conjugate stage) will reduce the overall number of animals used in cases where the same carrier protein lot is used for the preparation of more than one bulk conjugate.

## 9.3 Oral cholera vaccine

The mouse weight gain test currently used to assess the toxicity of oral cholera vaccine lots is considered to be insufficiently sensitive and of questionable relevance. The development and use of a more suitable and validated in vitro test (or combination of in vitro tests) is therefore

recommended. Examples include the potential use of the Y-1 adrenal cell assay for cholera toxin as a more specific test for residual specific toxicity. Such a test could be used either on a-lot-to-lot basis or to validate the production process *(150)*.

### 9.4 Whole cell pertussis vaccine

The mouse weight gain test currently used to assess the specific toxicity of whole cell pertussis vaccines is considered to be imprecise and non-specific, and manufacturers are encouraged to develop and use a validated in vitro test (or combination of in vitro tests) that targets specific potential toxins in the vaccine *(151, 152)*. Where levels of pertussis toxin in the whole cell pertussis vaccine are monitored, use of the HIST is not recommended due to the availability of an in vitro CHO cell assay which is recommended for this purpose *(153)*.

### 9.5 BCG vaccine

Current WHO recommendations include the use of guinea-pig tests to check for excessive dermal reactivity and the potential presence of virulent mycobacteria. However, manufacturers should instead develop and use validated in vitro assays, which in the case of testing for virulent mycobacteria might include molecular and/or cell culture methods.

### 9.6 Conclusions

Routine tests for specific toxicity are an essential part of the control strategy for a number of vaccines. Where scientifically relevant and suitably validated in vitro alternatives are available, it is strongly recommended that these are now implemented as replacements for any currently used in vivo methods. Where available, historic data on the sensitivity of the in vivo method for a particular toxin should be used for comparison against the alternative method as part of validation studies to avoid the need for additional animal studies. For some products, testing performed at later stages of the production process (for example on the final bulk) is redundant if assurance regarding safety has been obtained from testing performed at an earlier stage of the production process. Manufacturers and NRAs should critically review their control strategies to identify any tests that are potentially redundant and take steps to remove them.

## 10. Innocuity testing

The innocuity test (also referred to as the abnormal toxicity test or general safety test) was a previously recommended in vivo test carried out on the final product for the purpose of biological product licensing or quality control. Developed in the early 1900s, the test was originally intended to ensure the safe and consistent production of serum products, and later became a general safety test for detecting extraneous contaminants in all biological products *(154)*. Historically, the test has been included in WHO Recommendations, Guidelines and other guidance documents on vaccines and other biological products, and in pharmacopoeias worldwide. The test involved injecting the product into guinea-pigs and/or mice with lots

passing the test if no animal died or showed any signs of illness or relevant body weight changes within 7 days. The exact test design varied significantly between different pharmacopoeias and international requirements *(154)* and has long been under scrutiny for both its scientific value and relevance. Such scrutiny, together with the assurance provided following implementation of GMP, the use of validated manufacturing processes and appropriate quality control measures for biological products have now rendered the test expendable. As a result, NRAs and pharmacopoeias have removed, or are working to remove, the test from their respective requirements and monographs *(14, 155–158)*.

At its meeting in October 2018, the WHO Expert Committee on Biological Standardization reviewed the scientific rationale for performing the innocuity test for the purpose of marketing authorization and lot release of biological products. The Committee noted that current manufacturing processes, which incorporate GMP and comprehensive quality control measures, including in-process controls, provide more appropriate assurance than the innocuity test with regard to the quality and safety of vaccines and other biological products. Therefore, the Committee recommended the immediate discontinuation of any mention of the test in future WHO Recommendations, Guidelines and other guidance documents on biological products published in the WHO Technical Report Series. The Committee also recommended that the inclusion of this test in previously published such WHO documents be disregarded *(15)*. The Committee concluded that these recommendations represented a significant step towards increasingly science-based regulation and international regulatory convergence.

As part of subsequent efforts to promote wider awareness of these recommendations, and to facilitate the disregarding of any mention of the innocuity test in already published WHO documents on biological products, the Committee requested, at its meeting in October 2023, that a complete list of all such documents be appended to its future reports *(159)*. As of 2025, 34 such WHO documents were still current and are listed below in Appendix 1.

### 10.1 Conclusions

It is strongly recommended that manufacturers and NRAs remove any requirement for the innocuity test from the control strategy of biological products. In addition, any mention of this test appearing in the WHO Recommendations, Guidelines and other guidance documents on biological products listed in Appendix 1 should be disregarded.

## 11. Development and use of international and other biological reference standards

Where available, WHO biological reference preparations are the primary reference standards used worldwide to calibrate biological assays and improve the comparability of results obtained by different laboratories. Such reference preparations are typically assigned arbitrary units of biological activity following evaluation in multi-laboratory collaborative studies using one or more methods set out in regulatory guidelines and monographs. Consequently, any shift towards the replacement or removal of animal methods specified in such written standards may have implications for the use of some current and future reference preparations.

In cases where an animal method is replaced by a validated in vitro alternative for the purposes of quality control but continues to be used for nonclinical development and early research and development, the WHO reference preparation will need to be retained. In addition, in some situations, the use of the animal method may currently be necessary for the calibration of secondary standards. For example, heterogeneity in the glycosylation of therapeutic protein hormones across different products means that the relationship between the in vivo and in vitro assay results will differ for different products. In such cases, although the in vitro assay may be suitable for routine lot control, maintaining the link to in vivo bioactivity (and therefore product dosing) will require calibration of a manufacturer's in-house standard (representative of the product being tested) against the WHO or regional reference preparation, using the in vivo assay. These manufacturer's in-house standards can then be used in the in vitro assay to calibrate lots of working standard for the in vitro assay or used directly in the in vitro assay to measure bioactivity as part of routine quality control of lots of that manufacturer's product.

However, in other cases, the development of new WHO reference preparations can directly support the development, calibration and routine use of non-animal methods as part of the growing efforts being made to reduce reliance on animal testing for quality control. For example, in the rapidly developing field of molecular analysis, the establishment of the First WHO International Reference Panel for adventitious virus detection in biological products by high-throughput sequencing will facilitate the broader use of such advanced and highly sensitive non-animal technologies *(160)*. In addition, where the same WHO reference preparation can be used to support multiple assay types (for example, the Seventh WHO International Standard for rabies vaccine) their further characterization could allow for assay calibration and value assignment using one or more non-animal methods. Even in cases where an existing WHO reference preparation used to calibrate an in vivo assay is not considered suitable for calibrating an in vitro assay, it may still be useful as an assay control to help monitor and assess the performance of the replacement in vitro assay following its implementation.

## 12. Guidance for national regulatory authorities

NRAs have the responsibility to oversee access to high-quality, safe and efficacious vaccines and other biological products in their jurisdiction. In carrying out this role, they follow scientific and risk-based decision-making principles using, among other resources, published WHO guidance and the requirements of the relevant pharmacopoeia. The replacement of animal tests with alternative in vitro approaches for the quality control testing of vaccines and other biological products can result in more scientifically relevant and robust assurance of production consistency, reduce testing burdens and facilitate more rapid access to products.

With the rapid advances in technology now taking place, it is recognized that previously published WHO and other guidance may not always be aligned with current opportunities to implement improved methods. NRAs should therefore remain open to the use of alternative in vitro approaches that have been adequately validated and scientifically justified. NRAs should consider putting in place mechanisms to allow for the authorization of such approaches and should promote their consistent regulatory application. As the adequate assessment and critical review of any proposed new approach will necessitate a certain level of knowledge and

understanding of the underlying methodology, NRAs also have a responsibility to keep up to date with regard to the appropriate knowledge and expertise.

It should also be kept in mind that vaccines and other biological products are evaluated and used on a global scale. Promoting acceptance of alternative in vitro approaches to the routine quality control of such products through information and data exchange, cooperation and international alignment among regulatory counterparts around the world will be key to accelerating the implementation of validated and scientifically relevant non-animal testing approaches. Indeed, manufacturers may be reluctant to adopt such in vitro alternative approaches where they have not been accepted in all jurisdictions *(3)*. Cooperation and interaction at the global level between different regulatory authorities also offers the possibility of enhanced regulatory reliance and associated regulatory strengthening. In situations where an NRA may not yet have the necessary experience or expertise to conduct a robust evaluation, interaction with a reference regulatory authority and reliance on its findings and decisions can be an effective solution *(44)*. In addition, in the case of independent quality control testing by NCLs, reliance on the previous findings of tests carried out by another reference regulatory authority is also strongly recommended *(13)*.

## Authors and acknowledgements

The first draft of these WHO Guidelines was based on the initial review of WHO Recommendations, Guidelines and other guidance documents conducted by the NC3Rs expert working group led by Dr E. Lilley. The document was prepared by a drafting group comprising: Dr I. Feavers, consultant, United Kingdom; Dr T. Wu, Health Canada, Canada; Dr J. Joseph, Health Canada, Canada; Dr C. Milne, European Directorate for the Quality of Medicines & HealthCare, France; Dr P. Stickings, Medicines and Healthcare products Regulatory Agency, United Kingdom; Dr B. Kerscher, Paul-Ehrlich-Institut, Germany; Dr D. Lei, World Health Organization, Switzerland; Dr R. Levis, US Food and Drug Administration, USA; Dr W. Wongchana, Ministry of Public Health, Thailand; Dr M. Xu, National Institutes for Food and Drug Control, China; and Dr G. Raychaudhuri, US Food and Drug Administration, USA.

The resulting document was posted on the WHO Biologicals website for a first round of public consultation from December 2024 to January 2025. The second draft was then prepared by Dr I. Feavers, consultant, United Kingdom; Dr T. Wu, Health Canada, Canada; Dr J. Joseph, Health Canada, Canada; Dr C. Milne, European Directorate for the Quality of Medicines & HealthCare, France; Dr P. Stickings, Medicines and Healthcare products Regulatory Agency, United Kingdom; Dr B. Kerscher, Paul-Ehrlich-Institut, Germany; Dr D. Lei, World Health Organization, Switzerland; Dr W. Wongchana, Institute of Biological Products, Thailand; and Dr M. Xu, National Institutes for Food and Drug Control, China, incorporating comments received from regulatory authorities, manufacturers and other stakeholders as appropriate.

The third draft of the Guidelines was prepared by Dr I. Feavers, consultant, United Kingdom; Dr C. Milne, European Directorate for the Quality of Medicines & HealthCare, France; Dr T. Wu, Health Canada, Canada; Dr J. Joseph, Health Canada, Canada; Dr P. Stickings, Medicines and Healthcare products Regulatory Agency, United Kingdom; Dr B. Kerscher, Paul-Ehrlich-Institut, Germany; Dr D. Lei, World Health Organization, Switzerland;

Dr W. Wongchana, Ministry of Public Health, Thailand; and Dr M. Xu, National Institutes for Food and Drug Control, China following a WHO informal consultation held in Geneva Switzerland 27–28 February 2025 and attended by: Dr J.A. Cabral da Costa, National Institute for Quality Control in Health, Brazil; Dr I. Feavers, consultant, United Kingdom; Dr K. Ishii, National Institute of Infectious Diseases, Japan; Dr M. Iwaki, National Institute of Infectious Diseases, Japan; Dr J. Joseph, Health Canada, Canada; Dr J. Joung, Ministry of Food and Drug Safety, Republic of Korea; Dr B. Kerscher, Paul-Ehrlich-Institut, Germany; Dr E. Lilley, NC3Rs, United Kingdom; Dr C. Milne, European Directorate for the Quality of Medicines & HealthCare, France; Dr S. Morgeaux, L'Agence nationale de sécurité du médicament et des produits de santé, France; Dr E. Park, Ministry of Food and Drug Safety, Republic of Korea; Mr D. Ramondrana, National Quality Control Laboratory of Drug and Food, Indonesia; Dr D. Smith, International Alliance for Biological Standardization, Canada: Dr P. Stickings, Medicines and Healthcare products Regulatory Agency, United Kingdom; Dr L. Tesolin, Sciensano, Belgium; Dr T. Waddell, United Kingdom; Dr W. Wongchana, Ministry of Public Health, Thailand; Dr T. Wu, Health Canada, Canada; Ms L. Viviani, SciEthiQ Consulting, Italy; Dr M. Xu, National Institutes for Food and Drug Control, China; and Ms M. Bovenschulte, Dr E. Kim, Dr I. Knezevic, Dr D. Lei and Dr T.Q. Zhou, World Health Organization, Switzerland. *Representative of the Developing Countries Vaccine Manufacturers Network*: Dr P.K. Das, Biological E Limited, India. *Representative of the European Partnership for Alternative Approaches to Animal Testing*: Dr S. Shaid, GSK Vaccines GmbH, Germany. *Representatives of the International Federation of Pharmaceutical Manufacturers & Associations*: Dr S. Berhane, Switzerland, Dr E. Coppens, Sanofi, France and Dr S. Sheridan, Merck, United Kingdom.

Editorial review of the resulting document was then carried out by Dr T. Waddell, United Kingdom in accordance with WHO requirements for all documents appearing in the WHO Technical Report Series.

The resulting document (WHO/BS/2025.2485) was then posted on the WHO Biologicals website during July and September 2025 for a second round of public consultation. Twenty sets of comments were received from regulators, industry, individual experts and other stakeholders.

Further changes were made to document WHO/BS/2025.2485 by the WHO Expert Committee on Biological Standardization.

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# Appendix 1

## List of WHO documents for biological products published prior to 2018 in which any mention of the innocuity test (also known as the abnormal toxicity test or general safety test) should be disregarded

| Product | WHO document | Name of test as it appears in document |
|---|---|---|
| Dengue fever vaccines (live, attenuated) | Annex 2: TRS 979 | General safety |
| Diphtheria vaccines (adsorbed) | Annex 4: TRS 980 | Innocuity |
| DT-based combined vaccines | Annex 6: TRS 980 | [Control of final product] |
| Ebola vaccines | Annex 2: TRS 1011 | General safety (innocuity) |
| Hepatitis A vaccines (inactivated) | Annex 2: TRS 858 | General safety |
| Hepatitis B vaccines (recombinant) | Annex 4: TRS 978 | General safety (innocuity) |
| HFRS vaccines (inactivated) | Annex 2: TRS 848 | General safety |
| *Haemophilus influenzae* type b conjugate vaccines | Annex 1: TRS 897 | General safety (innocuity) |
| Human papilloma virus VLP vaccines | Annex 4: TRS 999 | General safety (innocuity) |
| Human interferons | Annex 3: TRS 786 | Innocuity |
| Influenza vaccines (inactivated) | Annex 3: TRS 927 | General safety (innocuity) |
| Japanese encephalitis vaccines (inactivated) | Annex 1: TRS 963 | General safety (innocuity) |
| Japanese encephalitis vaccines (live, attenuated) | Annex 7: TRS 980 | General safety |
| Malaria vaccines (recombinant) | Annex 3: TRS 980 | General safety |
| Meningococcal A conjugate vaccines | Annex 2: TRS 962 | General safety (innocuity) |
| Meningococcal C conjugate vaccines | Annex 2: TRS 924 | General safety (innocuity) |
| Meningococcal polysaccharide vaccines (unconjugated) | Annex 2: TRS 594<br>Annex 2 TRS 904 | Abnormal toxicity |
| MMR and combined vaccines (live) | Annex 3: TRS 840 | General safety |
| Pertussis vaccines (acellular) | Annex 4: TRS 979 | Innocuity |
| Pertussis vaccines (whole cell) | Annex 6: TRS 941 | General safety (innocuity) |
| Pneumococcal conjugate vaccines | Annex 3: TRS 977 | General safety (innocuity) |

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|---|---|---|
| Poliomyelitis vaccines (inactivated)<br>Amendment (requirement removed) | Annex 3: TRS 993<br>Annex 3: TRS 1024 | General safety<br>(innocuity) |
| Rabies vaccines (inactivated) | Annex 2: TRS 941 | General safety<br>(innocuity) |
| Rift Valley fever vaccines (inactivated) | Annex 4: TRS 673 | Innocuity |
| Smallpox vaccines | Annex 1: TRS 926 | General safety<br>(innocuity) |
| Snake antivenom immunoglobulins | Annex 5: TRS 1004 | Abnormal toxicity |
| Synthetic peptide vaccines | Annex 1: TRS 889 | Routine control |
| Tetanus vaccines (adsorbed) | Annex 5: TRS 980 | Innocuity |
| Tick-borne encephalitis vaccines (inactivated) | Annex 2: TRS 889 | General safety |
| Typhoid vaccines (live attenuated, Ty 21a, oral) | Annex 3: TRS 700 | Innocuity |
| Typhoid vaccines (Vi polysaccharide) | Annex 1: TRS 840 | Abnormal toxicity |
| Vaccines (stability evaluation of) | Annex 3: TRS 962 | General safety<br>Abnormal toxicity |
| Varicella vaccine (live) | Annex 1: TRS 848 | General safety |
| Yellow fever vaccines (live, attenuated) | Annex 5: TRS 978 | General safety |

DT = diphtheria and tetanus; HFRS = haemorrhagic fever with renal syndrome; VLP = virus-like particle; MMR = measles, mumps and rubella.