National Centre
for the Replacement
Refinement & Reduction
of Animals in Research

Review of animal testing requirements in WHO guidelines and recommendations for biologics

A proposal to implement
3Rs principles

NC3Rs report to WHO ECBS

October 2023

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Executive summary

1. This report describes the output of a project to review animal testing requirements within World Health Organization (WHO) guidance documents for the quality, safety and efficacy of vaccines and biological therapeutics. It includes evidence-based recommendations for the wider integration of post-approval release (i.e. batch release) testing strategies which have the potential to replace, reduce or refine animal use (termed 'the 3Rs') and improve access to products by the communities who need them most. All active guidance documents approved by the Expert Committee for Biological Standardization (ECBS) and posted on the WHO web pages were included in the review which aimed to identify:
   - All instances where animal test methods are recommended for batch release testing.
   - Instances where 3Rs strategies are already described within these documents.
   - Opportunities for inclusion of non-animal test methods or other 3Rs strategies where scientifically justified.
   - Barriers to the adoption of 3Rs strategies by manufacturers, National Regulatory Authorities (NRAs) and National Control Laboratories (NCLs).
2. This 3Rs audit was conducted independently of the WHO by the UK National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) and co-funded by the Bill and Melinda Gates Foundation. An international expert working group (EWG; see Annex 4) consisting of regulators, manufacturers, and other relevant stakeholders was assembled to help guide the project. The project was presented to ECBS in October 2019 Technical Report Series (TRS 1024, section 2.2.2) and a mid-project progress report was discussed at their meeting in April 2022 (TRS 1043, section 2.1.3). This final report will be presented to the ECBS in October 2023.

3. Eighty-one WHO guidance documents were reviewed. Sixty-three of these described animal testing methods that were used to assess the presence of adventitious agents, neurovirulence, potency, pyrogenicity and toxicity of vaccines and biological products before they are released on the market (see Annex 2). A focus group was established in each of these areas to review the methods described and to propose alternative text/phrasing to those sections in the guidelines. The aim of this process was to suggest revisions to the text of the relevant WHO guidance document to incorporate and/or encourage the adoption of established, validated non-animal testing methods where they currently exist and to allow the use of 3Rs approaches that may be developed and validated in the future. The following approaches to support these efforts were adopted during the review process:
   - Standardised language was recommended wherever possible.
   - Global stakeholders were engaged throughout the project so that their experiences and perspectives could be included in the recommendations contained in this report and to accelerate the eventual adoption of 3Rs approaches. Surveys of manufacturers and NRAs/NCLs were conducted in 2021 and 2022, respectively, and three regional workshops were held in 2022 (see paragraph 67).
4. In addition to producing suggested revisions to the text of WHO guidance documents, the following recommendations were also produced by the EWG:
   - The EWG has recommended that the WHO draft a position statement and guidance on the incorporation of 3Rs practices into batch release testing functions based on sound scientific principles (see Annex 7).
   - The EWG has also recommended that the ECBS consider drafting a manual for supporting adoption of 3Rs approaches specifically for endotoxin and pyrogenicity testing (see Annex 6).
   - The review process also identified opportunities where changes to current WHO ECBS practice could improve accessibility and utility of WHO guidance documents going forward.
   - The EWG fully endorses the content of this report and recommend that the suggestions contained herein are fully considered by WHO ECBS.

Introduction

5. Animal testing has long been integral to the development of biological therapeutics and vaccines. The use of animals can provide important information on potential toxicity, insights into mechanism of action, pharmacokinetics and dynamics, physiologic distribution, and potency. However, the use of these same methods is often continued, under the terms of a product licence, into the post-licensure phase of the product life cycle for the monitoring of product qualities, such as potency or safety, as part of their routine batch release. It has been estimated that, for human vaccines, more than 10 million animals a year are used worldwide for these purposes [1]. The use of such a large number of animals puts a significant financial burden on both manufacturers and NCLs, is time and resource intensive and the methods themselves can result in significant pain and distress to the animals. The time required for biological responses to manifest, and the inherent variability of many of the animal methods used in batch release and quality control testing can cause significant delays to the release of vaccines and biological therapeutics through requirements for repeating the assay when results are obtained outside of their specifications and the initiation of complex root cause investigations [2, 3]. There is the added risk of failing a product batch altogether through a false report when it may otherwise be suitable for safe and efficacious use.

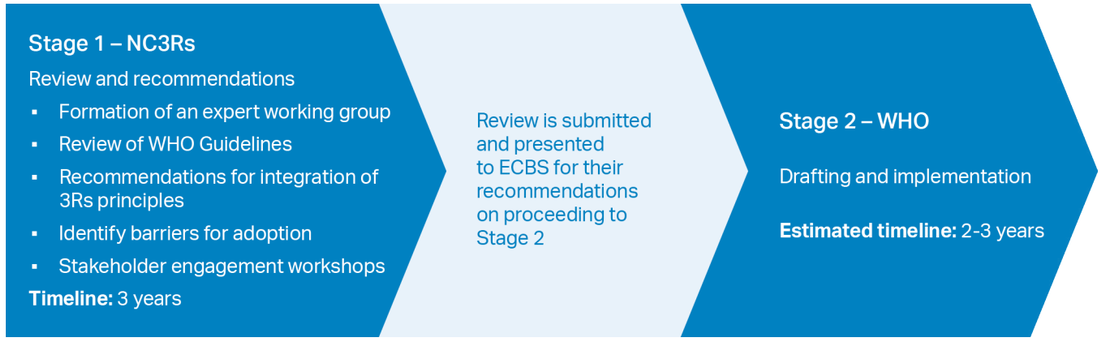
6. The 3Rs (Replacement, Reduction and Refinement of animals in research) are increasingly being applied to support more humane and scientifically robust animal research and as a framework for a scientific justification on the choice of testing methods adopted in many fields of research and testing across the biosciences. Over the last decade significant strides have been made to apply non-animal technologies in the quality control and batch release testing of biological products and to remove obsolete tests, such as the **general safety test**, from product release requirements. To date, the WHO, which establishes international guidelines and specifications relevant to the batch release testing of vaccines and biological therapeutics, has provided limited guidance which acknowledges the 3Rs and alternatives to animal testing. As a result, opportunities to embed the latest technologies into product quality control recommendations are being missed and animal tests that are expensive, often poorly predictive and variable continue to be used, causing delays in product release and possible increased costs. It is a timely and important step for WHO to better understand the extent of animal testing recommendations in its guidelines and recommendations, and to assess where there are already opportunities to apply non-animal testing approaches. This project aims to provide recommendations on where 3Rs approaches should be included within WHO guidance documents relevant to the quality control and release testing of biological therapeutics and vaccines. Having such text in these documents is expected to help the international harmonisation of testing requirements, improve robustness and precision of tests used in product safety and efficacy evaluation, thereby reducing the time and costs required to release biologics products onto the market.

7. Due to the size and complexity of the project, it was divided into two distinct stages (Figure 1). Stage One, led by the NC3Rs, reviewed the WHO biologics guidelines and developed recommendations for wider implementation of 3Rs approaches within them. Stage Two, implementation of the recommendations, would be the responsibility of the WHO Norms and Standards for Biological Products (NSB) team and dependent on the outcomes of Stage 1 and the recommendations of ECBS.

8. The project was endorsed by the WHO ECBS in October 2019 (TRS 1024, section 2.2.2). Additionally, the project was introduced at the WHO NCL Network meeting (Johannesburg, November 2019) and an International Alliance for Biological Standardisation (IABS) workshop on the 3Rs (Bangkok, December 2019) both with positive feedback. The project started in June 2020 and was co-funded by the NC3Rs and the Bill and Melinda Gates Foundation [Grant number 005622].

9. The project was completed in June 2023. This report, along with the publications in Annex 3, is the primary output that will be presented to the ECBS in October 2023.

Figure 1 – The stages of the project



The NC3Rs

10. The NC3Rs is an independent scientific organisation. It supports the global science base by driving and funding innovation and technological developments that replace or reduce the need for animals in research and testing and which lead to improvements in welfare where animals continue to be used. The Centre promotes robust and ethical scientific practice through collaborating with research funders, academia, industry, regulators, and animal welfare organisations, both in the United Kingdom and internationally. The NC3Rs has achieved success in the field of regulatory testing through a number of initiatives including the adoption of the Fixed Concentration Procedure (FCP) in acute inhalation studies of chemicals (OECD TG 433) [4, 5] and removal of the recommendations for conventional single dose rodent acute toxicity testing prior to first-in-human studies from the international pharmaceutical guidelines, ICH M3 [6, 7]. The NC3Rs has also supported the development and adoption of non-animal technology approaches in biological product development and testing, including *in vitro* biochemical and biological assays to replace the histamine sensitisation test for Adsorbed Pertussis Vaccines (Acellular Component) [8].

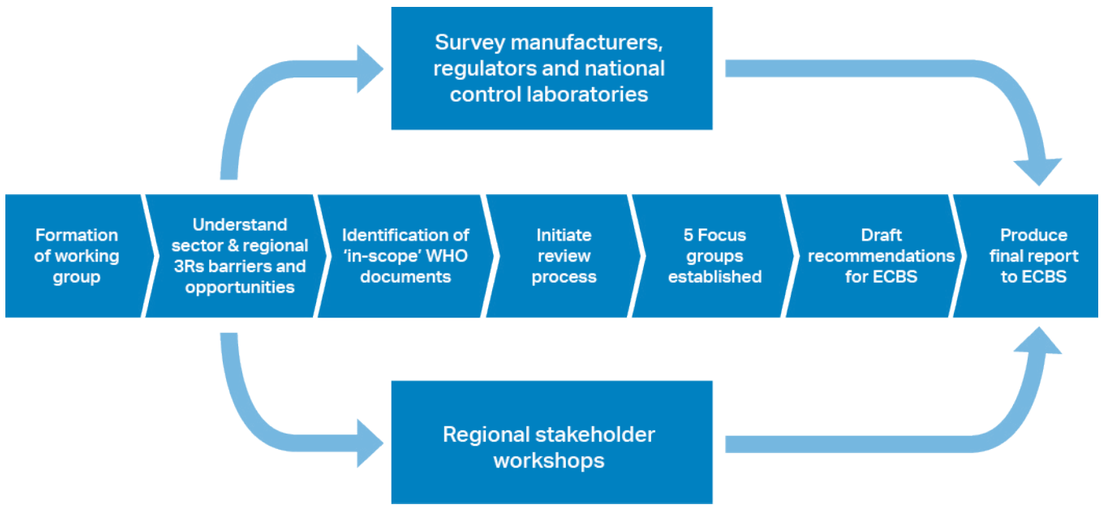
11. The NC3Rs was chosen to lead Stage One of the project in order to reduce any perceptions or risk of bias if WHO were to review its own documents, and to better ensure a proper inclusion of 3Rs principles about which NC3Rs has considerable knowledge and experience.

Project overview

12. Stage One of the project (Figure 2) addressed three major questions:
    - What is the extent of animal testing included within the collection of WHO recommendations for biologics and are there validated alternative methods that could replace these?
    - Would a WHO guideline for the adoption of 3Rs principles into the quality control and batch release testing of licensed vaccines and biological therapeutics be useful for harmonisation of non-animal technologies and for guidance to WHO member states?
    - What are the barriers that are hindering the adoption of 3Rs principles by manufacturers, NRAs and NCLs?

13. In addition to the formal review of WHO guidelines, the project supported the attitudinal and behavioural changes required when adopting new regulatory approaches and processes. This was achieved via continuous stakeholder engagement with NRAs, NCLs, biological therapeutics and vaccines manufacturers, WHO collaborating centres, research funders, academic researchers and small companies developing 3Rs models. This provided valuable opportunities to share project outputs with relevant communities and to learn from them what potential barriers exist and what strategies to include in the recommendations that could help overcome these and accelerate their integration in WHO guidelines.

Figure 2 – Flow chart describing the key features of the project



14. An EWG (see Annex 4) was established to help oversee this project. The main focus of the EWG was to provide professional input into the evaluation of WHO guidelines from 3Rs and regulatory perspectives and to help develop the recommendations to ECBS. Sub-groups of the EWG were established as necessary to deliver more specific, focused tasks including organising project workshops, writing project reports/manuscripts, and drafting the recommendations for ECBS. EWG members were invited from NRAs, NCLs, WHO biologics collaborating centres, vaccines and biological therapeutics manufacturers and relevant industry trade bodies (e.g. Developing Countries Vaccine Manufacturers Network; DCVMN, and International Federation of Pharmaceutical Manufacturers and Associations; IFPMA).

15. In addition to this final report, a number of publications have been produced during the project. These, and associated dissemination activities are listed in Annex 3.

Project scope

16. Based on input from ECBS and the working group, the project scope was established to include:
    - The review of all current WHO written and measurement standards relevant to the regulation or control of human biological therapeutics and vaccines and under the purview of ECBS.
    - All 3Rs (not just replacement).
    - Methods used in the post-licensure control of biological therapeutics and vaccines.
    - Identification of possible barriers towards adopting or implementing 3Rs strategies in the quality control and lot release testing of biological therapeutics and vaccines.
    - Development of scope and process for Stage Two.

17. The following were not in scope for the project:
    - The development or validation of 3Rs methods.
    - Confidential documents, or any guidelines, recommendations, or other documents which are not available within the public domain.
    - Animal testing encompassed within WHO documents that are outside of the purview of the ECBS (e.g. international pharmacopoeia).
    - The evaluation of non-WHO standards, guidelines, or regulations, outside of the purposes for providing examples and/or suggestions.
    - Non-constructive criticisms of WHO, its member states, and/or their regulatory processes.
    - Ethical review of the use of animals in the control of biological therapeutics and vaccines.
    - Drafting of full revisions of existing guidelines and recommendations.
    - Animal testing or methods used for research, exploratory studies.

The 3Rs and their scientific benefits

18. The 3Rs were originally described in 1959 by Russell and Burch in their seminal book 'The Principles of Humane Experimental Technique [9]'. While some would argue that application of these principles to research that uses animals is an ethical issue, there are clear scientific and economic advantages to the application of the 3Rs. This project was established on the basis that enhancing the application of the 3Rs in WHO guidelines would improve the quality and reproducibility of quality control, batch release testing of vaccines and biological therapeutics and that this would result in better control of, and faster access to, products by those that need them most.

19. For this project, and in particular the review process, the 3Rs are defined as follows:

Replacement

- Replacement refers to technologies or approaches which directly replace or avoid the use of animals in experiments where they would otherwise have been used. For many years research animals have been used to answer important scientific questions including those related to human health. Animal models are often costly and time-consuming and depending on the research question present scientific limitations, such as poor relevance to human biology. Alternative models can address some of these concerns. Within the last two decades, advances in science and technology have meant that there are now realistic opportunities to replace the use of animals. In recent years, the term new approach methodologies (NAMs) has been adopted by the bioscience sector specifically to describe non-animal technologies for use in assessing chemical or drug toxicity.
- Replacement can be divided into two categories, full and partial replacement:
  - Full replacement refers to methods that avoid the use of animals for research and testing purposes. It includes the use of human volunteers, tissues and cells, molecular methods, mathematical and computer models, and established cell lines – often referred to collectively as non-animal technologies. The MAT (monocyte activation test) assay (using human peripheral blood mononuclear cells; PBMCs) for pyrogenicity testing is an example of full replacement.
  - Partial replacement includes the use of some animals that, based on current scientific thinking, are not considered capable of experiencing suffering. This includes invertebrates such as *Drosophila*, nematode worms and social amoebae, and immature forms of vertebrates. Partial replacement also includes the use of primary cells (and tissues) taken from animals killed solely for this purpose. The LAL (Limulus Amebocyte Lysate) assay (using blood from horseshoe crabs) for endotoxin testing is an example of partial replacement.

- It is widely acknowledged [3, 10-12] that, compared to animal methods, *in vitro* assays are less variable, less time and resource consuming and are better suited for use in a control strategy to ensure consistent production of safe and effective batches of vaccines and biological therapeutics.

Reduction

- Reduction refers to methods that minimise the number of animals used per experiment or study consistent with the scientific aims. It is essential that studies with animals are appropriately designed and analysed to ensure robust and reproducible findings. Reduction also includes methods which allow the information gathered per animal in an experiment to be maximised in order to reduce the use of additional animals. Sharing data and resources (e.g. animals, tissues and equipment) between research groups and organisations can also contribute to reduction. Animal assays should always be optimised to deliver robust data, to enable clear decisions to be made and to ensure that studies do not need to be repeated, requiring additional animal use. The single dilution assay for potency testing (e.g. for diphtheria and tetanus vaccines), rather than an assay requiring multiple groups of differing dilutions, is an example of reduction.

Refinement

- Refinement refers to methods that minimise the pain, suffering, distress or lasting harm that may be experienced by laboratory animals, and which improve their welfare. Refinement applies to all aspects of animal use, from their housing and husbandry to the scientific procedures performed on them.
- Evidence suggests that pain and suffering can alter an animal's behaviour, physiology and immunology [13-15]. Such changes can lead to variation in experimental results that impairs both the reliability and repeatability of studies. Examples of refinement include ensuring the animals are provided with housing that allows the expression of species-specific behaviours, using appropriate anaesthesia and analgesia to minimise pain, training animals to cooperate with procedures to minimise any distress and the use of early humane endpoints instead of lethality endpoints in safety or potency assays. A specific example of refinement relevant to this project would be refinement of potency assays by replacing a challenge step with analysis of immune response by serology.

WHO TRS review and recommendations to ECBS

Summary of the WHO TRS review process

20. The main task of this project, the review of the WHO guidance documents published as annexes to the ECBS meeting reports (published in WHO TRS documents), was initiated in January 2021. This followed consultation with the working group in November 2020 where a proposal was presented and approved for the review process. An initial triage of the TRS documents available on the WHO website[1] identified 81 documents that were considered to be in scope for this project. A list of the documents that were reviewed can be found in Annex 2. To facilitate the review process, the documents were divided into four groups reflecting the nature of the products being described. These groups were:
    - Viral vaccines.
    - DTP (products that contain Diphtheria, Tetanus and/or Pertussis components) vaccines.
    - Other bacterial vaccines (excluding DTP vaccines).
    - Other biological therapeutics and general guidelines.

21. Working group members were selected to review documents within these groups based on their expertise and preference. The review group allocations are included in the EWG membership table in Annex 4.

22. Each group was allocated relevant WHO TRS documents and each document was reviewed by at least two group members (most documents were reviewed by three group members). Reviewers were provided with a review form and asked to record the following information as they reviewed each TRS document:
    - Any mention of animal tests related to quality control, batch or lot release.
    - The section and page number for each test.
    - Any mention of the 3Rs or language which could be considered 3Rs relevant.
    - Any possible 3Rs approach that could be applied.

23. All responses were collated and reviewed for consistency and entered into a Microsoft Excel database. Any discrepancies or disagreement were discussed among the group members to achieve consensus.

[1] www.who.int/teams/health-product-policy-and-standards/standards-and-specifications/trs-publications-listing
www.who.int/teams/health-product-and-policy-standards/standards-and-specifications/vaccine-standardization

Review output

24. The review identified 63 WHO TRS documents that contained animal tests and/or 3Rs information. Within these 63 documents, animal tests or references to the 3Rs that were in scope for the review were identified on 351 separate occasions by the review groups. Each of these was inputted into the database as a separate line for further analysis.

25. The animal test methods identified as being 'in scope' were further subdivided (based on the most commonly identified assays/tests) into five broad categories:
    - Adventitious agents testing.
    - Neurovirulence testing.
    - Pyrogen and endotoxin testing.
    - Potency and immunogenicity testing.
    - Specific toxicity testing.

26. Five focus groups, aligned to the categories above, were formed to review each line in the database. Each group was tasked with reviewing the original text describing the animal method or 3Rs language and to develop alternative text more inclusive of contemporary 3Rs information. The focus groups were made up from members of the working group and nominated additional experts (see Annex 5 for membership). Annex 1 is a table of all 351 animal tests or examples of 3Rs language that was identified in the review and the proposed alternative text drafted by the focus groups.

27. The review process identified numerous instances where the language used to describe the same test varied significantly between guidelines, often reflecting the knowledge and views of the WHO drafting group that wrote each TRS document at the time. The example below uses endotoxin or pyrogenicity testing for vaccines to highlight this:

Text related to pyrogenicity testing	TRS document
A test that has been found to be suitable for the current vaccine involves injection into the ear vein of rabbits.	Requirements for Vi polysaccharide typhoid vaccine; TRS 840 Annex 1 (1994).
The endotoxin content of the purified polysaccharide should be determined and shown to be within limits agreed by the national control authority in order to ensure that any pyrogenic activity of the final product is acceptable. Less that 10IU of endotoxin per ug of polysaccharide when measured by a LAL test can be achieved. Alternatively, polysaccharide preparations should pass the rabbit pyrogenicity test.	Recommendations for the production and control of haemophilus influenzae type b conjugate vaccines; TRS 897 Annex 1 (2000).
The vaccine in the final container should be tested for pyrogenic activity by intravenous injection into rabbits or by a Limulus amoebocyte lysate (LAL) test, which should be validated for this purpose.	Recommendations to assure the quality, safety and efficacy of group A meningococcal conjugate vaccines; TRS 962 Annex 2 (2011).
Each final lot should be tested for pyrogenic substances. The test procedures should be approved by the national regulatory authority.	Recommendations for Japanese encephalitis vaccine (inactivated) for human use; TRS 963 Annex 1 (2011).
Each final lot should be tested for pyrogenic substances. Where appropriate, tests for endotoxin – for example, the limulus amoebocyte lysate (LAL) test – should be performed. However, where there is interference in the test (for example, from the adjuvant) a test for pyrogens in rabbits should be performed. A suitably validated monocyte-activation test may also be considered as an alternative to the rabbit pyrogen test. The test is conducted until consistency of production is demonstrated, subject to the agreement of the NRA.	Recommendations to assure the quality, safety and efficacy of recombinant hepatitis E vaccines; TRS 1016 Annex 2 (2019).

28. The EWG felt strongly that one of the tasks of the review would be to draft standardised language wherever possible which integrated 3Rs opportunities and to recommend to ECBS that standardised language be considered in the drafting of future guidance documents.

General safety test

29. A group focused on the General Safety test (GST, also known as the abnormal toxicity test; ATT, or the test for innocuity) was not established. This is because the WHO has recognised the test is not scientifically justifiable and no longer recommends it for the quality control and batch release testing of any biological product (TRS 1016, 2019 page No 32-33 [16]). However, the review process did highlight that, of the 63 documents that reference animal methods for the batch release testing of vaccines and biological products, the GST was mentioned 38 times in 34 individual guidance documents (see **paragraphs 86 to 90**). It is clear from our **stakeholder engagement** activities and recent publications [17, 18], that the GST is still being performed and the continued presence of the test in these guidance documents may, in part, explain why the test continues to be used. The working group recommends that these guidelines be updated as soon as possible to remove all references to this test completely.

Focus group output and recommendations for ECBS

Overarching principles

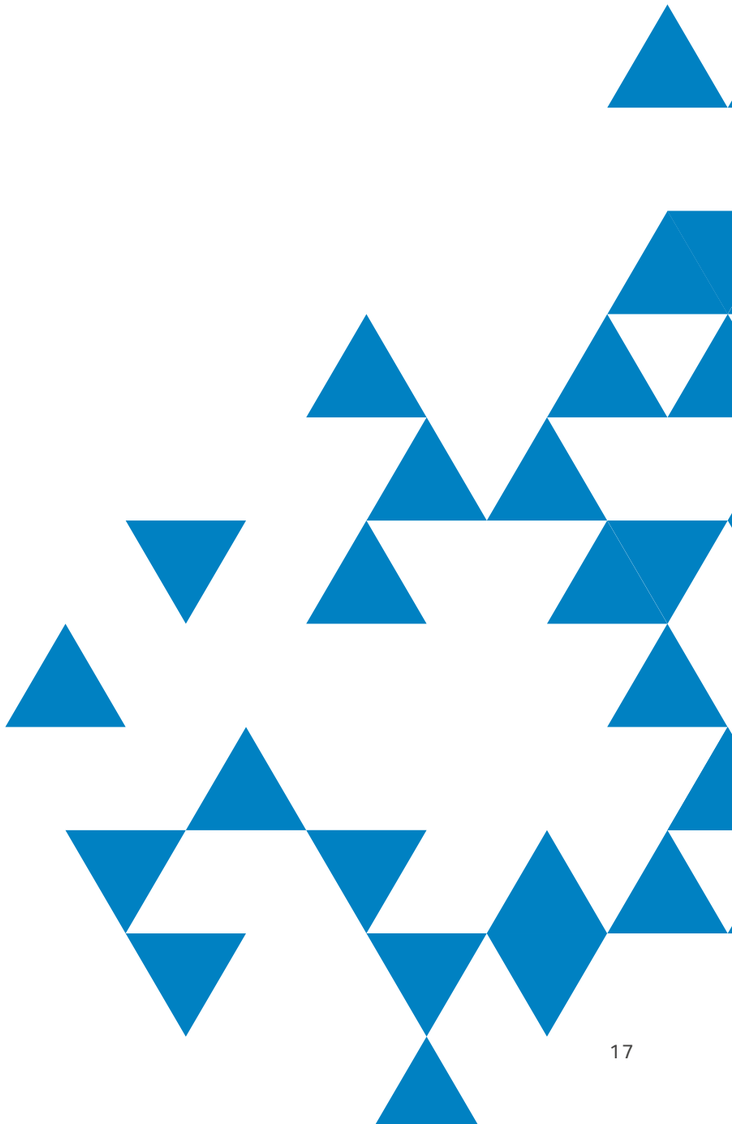
30. Six overarching principles were established to support the review process:
    - A risk-based approach should be applied to quality control and batch release testing.
    - Recommended alternative text should focus on a change in emphasis towards promotion of established, validated non-animal technology test methods wherever possible.
    - Where non-animal methods are not yet established or validated, the recommended alternative text should be drafted in such a way as to allow the use of non-animal technology test methods that may be developed and validated in the future.
    - When it is not practical or possible to apply an alternative approach, the recommended alternative text should still allow current *in vivo* test methods but, wherever possible, detail around specific models and species should be removed so as to allow easier adoption of test methods which better incorporate the 3Rs principles.
    - Deletion of current *in vivo* test methods would only be recommended when supported by robust scientific evidence which demonstrates a lack of relevance and/or redundancy.
    - Wherever possible, standardised language should be drafted.

31. Whilst the working group fully support robust application of the 3Rs, they recognised the challenge for manufacturers of existing products and especially for older 'legacy' products to change established and currently licenced test approaches.

32. The working group recognised that WHO would likely require establishment of their own drafting groups to re-write individual TRS documents and that the inclusion of any recommendations would require they go through proper WHO processes as is done for any other amendments or revisions to guidance document texts. Therefore, the proposed alternative texts provided in this report are examples of how the 3Rs may be incorporated into each of the documents that were reviewed in this project.

33. Each focus group consisted of internationally recognised experts in the manufacture, testing and regulation of vaccines and biological therapeutics. As such, the working group fully endorses the proposed alternative text as being appropriate to promote greater implementation of the 3Rs principles whilst maintaining robust quality control, batch release testing. The working group is confident that where non-animal technology testing approaches are suggested, these will be equally or more scientifically relevant, less variable and better suited for use in a control strategy than the animal-based testing approaches used currently.

34. Annex 1 contains a comprehensive table of all the animal tests or 3Rs language identified during the review process. The table in Annex 1 is arranged alphabetically by product. However, a Microsoft Excel version of the table is provided alongside this report which can be filtered and sorted as required. The NC3Rs will host a dedicated web resource that will be published alongside this report. The resource will include a searchable database comprised of the data from the spreadsheet.



Adventitious agents testing

35. Identifying an adventitious agent in a biological medicinal product has been of concern to regulatory agencies, manufacturers and public health officials since the early 1900s when 13 children died of Tetanus from contaminated Diphtheria antitoxin [19]. Adventitious agents are defined as microorganisms that may have been unintentionally introduced during the manufacturing process of a biological medicinal product. These include bacteria, fungi, mycoplasma/spiroplasma, mycobacteria, rickettsia, protozoa, parasites, transmissible spongiform encephalopathy (TSE) agents and viruses. Adventitious agents could be inadvertently introduced into a vaccine or biological therapeutic through starting materials used for production, such as cell substrates, porcine trypsin, bovine serum, or any other source materials of animal or human origin. Historically, both *in vitro* and *in vivo* tests have been performed to detect contamination with adventitious agents [20]. However, recent analysis has indicated that modern *in vitro* assays, including broad molecular methods, are more sensitive and selective than the *in vivo* assays and should be used where possible [21-23].

36. The adventitious agents focus group reviewed 54 entries in the database that related to specific animal tests. These were subdivided into the following categories:

    - Mycobacteria.
    - Haemadsorbing viruses.
    - Avian viruses.
    - Other adventitious viruses.

37. The focus group concluded that it was possible to draft a single paragraph for each of these four categories and that the proposed alternative text could be used to substitute the current text in all of the TRS guidance documents that were reviewed.

Mycobacteria

Table 1 – TRS documents recommending testing for adventitious mycobacteria

WHO guideline title	TRS	Year	Product	Page #	Section #
Recommendations to assure the quality, safety and efficacy of BCG vaccines	979 Annex 3	2013	BCG vaccine	148	A.3.2.5
Recommendations to assure the quality, safety and efficacy of Japanese encephalitis vaccines (live, attenuated) for human use	980 Annex 7	2014	Japanese encephalitis vaccines (live, attenuated)	423 430	A.3.2.5.4 A.4.2.4.1
Guidelines on the quality, safety and efficacy of dengue tetravalent vaccines (live, attenuated)	979 Annex 2	2013	Dengue fever vaccines	69	A.3.2.5.3

38. Proposed new text:

    A test for the absence of virulent mycobacteria should be performed. Where available and appropriately validated, an in vitro test should be used (for example a validated nucleic acid amplification test or culture method). If in vitro assays are not available or appropriate, a suitable compendial in vivo test may be used.

39. This proposed new text uses language that is similar to that found in more recently updated TRS documents (e.g. 980 Annex 7; 2014), avoids mention of specific *in vivo* tests (a specific guinea-pig assay is described in some older TRS documents) and highlights the potential of molecular biological techniques and/or culture approaches that can achieve comparable results.

Haemadsorbing viruses

Table 2 – TRS documents recommending testing for adventitious haemadsorbing viruses

WHO guideline title	TRS	Year	Product	Page #	Section #
Recommendations to assure the quality, safety and efficacy of Japanese encephalitis vaccines (live, attenuated) for human use	980 Annex 7	2014	Japanese encephalitis vaccines (live, attenuated)	427	A.4.1.1
Guidelines on the quality, safety and efficacy of Ebola vaccines	1011 Annex 2	2018	Ebola vaccines	114	A3.1.1.1
Recommendations for the production and control of influenza vaccine (inactivated)	927 Annex 3 1007 Annex 8	2005 2017	Influenza vaccines (inactivated)	115	A.3.3.3.1
Proposed requirements for Rift Valley Fever Vaccine (inactivated) for human use	673 Annex 4	1981	Rift Valley Fever vaccine	112	3.2.1
Recommendations to assure the quality, safety and efficacy of enterovirus 71 vaccines (inactivated)	1030 Annex 3	2021	Enterovirus 71 vaccine (inactivated)	178	A.4.1.2
Recommendations for inactivated rabies vaccine for human use produced in cell substrates and embryonated eggs	941 Annex 2	2007	Rabies vaccine	100	A.4.1.1.1
Requirements for hepatitis A vaccine (inactivated)	858 Annex 2	1995	Hepatitis A vaccine (inactivated)	43	A.4.1.1
Recommendations to assure the quality, safety and efficacy of poliomyelitis vaccines (inactivated)	993 Annex 3 1024 Annex 3	2015 2020	Poliomyelitis vaccines (inactivated)	108	A.4.1.2
Recommendations to assure the quality, safety and efficacy of poliomyelitis vaccines (oral, live, attenuated)	980 Annex 2	2014	Poliomyelitis vaccines (oral, live, attenuated)	67	A.4.1.2
Requirements for varicella vaccine (live)	848 Annex 1	1994	Varicella vaccine	30	A.4.3.1
Guidelines on the quality, safety and efficacy of dengue tetravalent vaccines (live, attenuated)	979 Annex 2	2013	Dengue fever vaccines	72	A.4.1.1
Guidelines to assure the quality, safety and efficacy of live attenuated rotavirus vaccines (oral)	941 Annex 3	2007	Rotavirus vaccine	149	A.4.1.1
Recommendations to assure the quality, safety and efficacy of recombinant human papillomavirus virus-like particle vaccines	999 Annex 4	2016	HPV	170	A.4.2.1.1
Requirements for measles, mumps, rubella vaccines and combined vaccine (live)	840 Annex 3	1994	MMR	113 128 144	A.4.3.1 A.4.3.1 A.4.3.1
Requirements for tick-borne encephalitis vaccine (inactivated)	889 Annex 2	1999	Tick-borne encephalitis vaccine (inactivated)	52	A.4.1.2

40. Proposed new text:

At the end of the observation period a fraction of culture comprising not less than 25% of the total should be tested for the presence of haemadsorbing viruses, using red blood cells from guinea-pig or other suitable red blood cells. It is not necessary to use red blood cells from multiple species. If the red blood cells have been stored prior to use in the haemadsorption assay, the duration of storage should not have exceeded 7 days and the temperature of storage should have been in the range of 2–8 °C.

41. This proposed new text simplifies and standardises the variable language found in the documents that were reviewed. Some documents mentioned the use of blood cells from a wide range of animal species, and it was felt that it was unclear whether all or some of these species were required to be used, potentially increasing unnecessarily the number of animals used overall. The focus group proposed that in most cases guinea-pig blood cells would be acceptable for this test but recognised that manufacturers may have used blood cells from another specific species (including human) and have drafted the proposed new text to allow this.

Avian viruses

Table 3 – TRS documents recommending animal testing for avian adventitious agents

WHO guideline title	TRS	Year	Product	Page #	Section #
Recommendations to assure the quality, safety and efficacy of influenza vaccines (human, live attenuated) for intranasal administration	977 Annex 4	2013	Influenza vaccines (live attenuated)	188	A.5.3.4.2
Recommendations for inactivated rabies vaccine for human use produced in cell substrates and embryonated eggs	941 Annex 2	2007	Rabies vaccine	98 101	A.3.2.1.2 A.4.1.1.4

42. Proposed new text:

Each virus seed lot propagated in avian tissues and each virus harvest propagated in primary avian tissues should be tested for avian viruses if the risk assessment, approved by the NRA, indicates that this test provides a risk mitigation taking into account the overall testing package. Relevant culture methods and/or molecular biology or broad molecular methods approved by the NRA should be part of the overall testing package. Animal testing (including fertilised SPF eggs) may only be used to qualify virus seed lots if the risk assessment indicates that such testing provides a risk mitigation taking into account the overall testing package. Animal testing is not performed on virus harvest for routine batch release.

43. The new proposed text for avian viruses (for vaccines derived from virus seeds propagated in eggs) is based on a risk-based approach to testing. If the risk of adventitious agents is identified during preclinical development then a strategy should be developed to mitigate this risk through an appropriate testing approach. *In vivo* testing should be avoided unless the risk assessment indicates that it is essential for risk mitigation.

Other adventitious viruses

Table 4 – TRS documents recommending animal-based adventitious agents testing

WHO guideline title	TRS	Year	Product	Page #	Section #
Recommendations for Japanese encephalitis vaccine (inactivated) for human use (revised 2007)	963 Annex 1	2011	Japanese Encephalitis vaccine (inactivated)	71	A.3.2.4.3
Recommendations to assure the quality, safety and efficacy of Japanese encaphalistis vaccines (live, attenuated) for human use	980 Annex 7	2014	Japanese encephalitis vaccines (live, attenuated)	423	A.3.2.5.5
Proposed requirements for Rift Valley Fever vaccine (inactivated) for human use	673 Annex 4	1981	Rift Valley Fever vaccine	114	3.4.1
Recommendations for inactivated rabies vaccine for human use produced in cell substrates and embryonated eggs	941 Annex 2	2007	Rabies vaccine	97	A3.2.1.2.2
Guidelines on the quality, safety and efficacy of respiratory syncytial virus vaccines	1024 Annex 2	2020	Respiratory syncytial virus vaccines	119 124	A.3.3.3.4 A.3.4.2.1.3
Recommendations to assure the quality, safety and efficacy of poliomyelitis vaccines (inactivated)	993 Annex 3 1024 Annex 3	2015 2020	Poliomyelitis vaccines (inactivated)	103	A.3.1.3.2.1
Recommendations to assure the quality, safety and efficacy of live attenuated yellow fever vaccines	978 Annex 5	2013	Yellow fever vaccines	253	A.4.2.2.4
Haemorrhagic fever with renal syndrome (HFRS) vaccines (inactivated)	848 Annex 2	1994	Haemorrhagic fever vaccines	60 61	A.3.1.4 A.3.2.3

WHO guideline title	TRS	Year	Product	Page #	Section #
Guidelines to assure the quality, safety and efficacy of live attenuated rotavirus vaccines (oral)	941 Annex 3	2007	Rotovirus vaccine	148	A.3.2.3.4
Recommendations to assure the quality, safety and efficacy of recombinant human papillomavirus virus-like particle vaccines	999 Annex 4	2016	HPV	166	A.3.2.1.3
Requirements for measles, mumps, rubella vaccines and combined vaccine (live)	840 Annex 3	1994	MMR	113 129 145	A.4.3.2 A.4.3.2 A.4.3.2
Requirements for human interferons prepared from lymphoblastoid cells	786 Annex 3	1989	Human interferons	83	4

44. Proposed new text:

    A strategy for testing adventitious viruses in vaccines must be developed based on a risk assessment. Relevant culture methods and/or specific molecular biology or broad molecular methods should be part of the overall testing package with the agreement of the NRA. In vivo tests may only be used if the risk assessment indicates that this test provides an additional risk mitigation taking into account the overall testing package.

45. The new proposed text for adventitious agents that do not fall into the three other categories in this section is also grounded in a risk-based approach to testing. If the risk of adventitious agents is identified during development, then a strategy should be developed to mitigate this risk through an appropriate testing approach. The focus group emphasised the advantages of *in vitro* approaches including cell culture and molecular biology methods. However, an *in vivo* test may be used if the risk assessment indicates that such an approach is essential for risk mitigation.

Neurovirulence testing

46. Many viruses, such as measles, mumps and polio, are associated with severe neurological disease. Unlike most other acute health effects from viral infection, neurological damage can be long-term and life-altering for patients [24, 25]. For this reason, there is a critical need for vaccine development to be rapid without compromising safety. The testing of live attenuated viral vaccines for neurovirulence has been a requirement of many regulatory authorities for many years.

47. Neurovirulence testing was the most challenging area with respect to a lack of validated non-animal technology test methods. The risk of neurovirulence is product specific and consequently non-animal technology must also be developed and validated specifically for each product. The review process identified several vaccines where neurovirulence testing was recommended for quality control and batch release testing:

Table 5 – TRS documents recommending animal-based neurovirulence testing

WHO guideline title	TRS	Year	Product	Page #	Section #
Recommendations to assure the quality, safety and efficacy of Japanese encaphalistis vaccines (live, attenuated) for human use	980 Annex 7	2014	Japanese encephalitis vaccines (live, attenuated)	425 426 433	A.3.2.5.6.1 A.3.2.5.6.2 A.4.4.2.3
Recommendations for the production and quality control of smallpox vaccine (revised 2003)	926 Annex 1	2004	Smallpox vaccine	52	A.3.3.5.1
Guidelines for the safe production and quality control of poliomyelitis vaccines	1016 Annex 4	2019	Poliomyelitis vaccines	239	13.3
Recommendations to assure the quality, safety and efficacy of poliomyelitis vaccines (inactivated)	993 Annex 3 1024 Annex 3	2015 2020	Poliomyelitis vaccines (inactivated)	115	A.4.4.2.7.2
Recommendations to assure the quality, safety and efficacy of poliomyelitis vaccines (oral, live, attenuated)	980 Annex 2	2014	Poliomyelitis vaccines (oral, live, attenuated)	65 75	A.3.2.4.2 A.4.4.7.2

WHO guideline title	TRS	Year	Product	Page #	Section #
Requirements for Varicella vaccine (Live)	848 Annex 1	1994	Varicella vaccine	29	A.4.2.1
Guidelines on the quality, safety and efficacy of dengue tetravalent vaccines (live, attenuated)	979 Annex 2	2013	Dengue fever vaccines	70 71	A.3.2.5.5 A.3.2.5.5
Requirements for measles, mumps, rubella vaccines and combined vaccine (live)	840 Annex 3	1994	MMR	105 111 127 143	1 A.4.2.1 A.4.2.1 A.4.2.1
Recommendations to assure the quality, safety and efficacy of live attenuated yellow fever vaccines	978 Annex 5	2013	Yellow fever vaccines	254	A.4.2.2.5

48. Of these, the only validated non-animal neurovirulence assay is the MAPREC (mutant analysis by PCR and restriction enzyme cleavage) [26, 27] assay for oral poliovirus (Sabin) vaccines. Given the lack of current validated non-animal technology approaches for neurovirulence testing, the focus group chose to develop a guidance statement setting out principles whereby a risk-based approach could be used to avoid the need for neurovirulence testing for existing and future products.

49. Proposed new text:

    The potential neurovirulence of a new vaccine strain should be assessed during preclinical development and a risk analysis carried out based on available scientific data and information. If molecular consistency has been demonstrated during characterisation, then assessment of neurovirulence may be omitted for subsequent viral seed lots and/or routine manufacturing.

    For existing products where animal neurovirulence testing is currently prescribed, this test can be waived when safety and genetic stability of the product are sufficiently assured. This can be established by historical / (pre-) clinical, and pharmacovigilance data, and by data generated with nucleic acid amplification and sequencing techniques, to support the molecular consistency of the virus and for the establishment of a link between genetic sequences and in vivo phenotypes.

    For all products, a risk-based approach should be performed taking into consideration the genetic features and molecular consistency of the strain (sequence evaluated at different manufacturing steps, determined with traditional or new sequencing technologies) and the nature of the vaccine (attenuated, chimeric, genetically modified), to assess whether a neurovirulence assay is required and what animal model is most suitable. If an in vivo assay is scientifically justified, it should be established at what level (Master Seed Lot, Working Seed Lot, monovalent bulk) the test should be performed to avoid unnecessary duplication.

50. Clearly, there is a need for further research into novel non-animal technology approaches for neurovirulence testing and the working group would encourage initiatives to support this. Some progress has been made [24, 28-30] and the working group encourages WHO to monitor this area of research and incorporate these methods within their guidelines as soon as they have been validated.

Pyrogen and endotoxin testing

51. Testing of endotoxin and non-endotoxin pyrogens is an area of considerable achievement with respect to the development of non-animal technology testing approaches. Traditionally, pyrogenicity has been tested using the rabbit pyrogen test (RPT), first introduced in 1942. However, this assay has been widely reported to be prone to both false positive and false negative results, to have significant technical limitations and to be poorly predictive of responses in humans [31-34]. In 1964, the observation that endotoxin causes coagulation of the haemolymph of the horseshoe crab *Limulus polyphemus* was first reported. This eventually led to the establishment of the limulus amoebocyte lysate (LAL) test (in some regions, *Tachypleus gigas* or *Tachypleus tridentatus* are used in a tachypleus amebocyte lysate; TAL assay). Despite not completely replacing the RPT (due to an inability to detect non-endotoxin pyrogens), this was still a significant development as it reduced the need to use rabbits. However, use of the LAL/TAL test raises significant ethical concerns over horseshoe crab welfare and the preservation of their ecosystem [35].

52. The monocyte activation test (MAT) was first described in the 1980s as an *in vitro* pyrogen test and was validated as a potential replacement for the RPT in the early 2000s [36]. In principle, the MAT can be used as a universal pyrogen test (for both endotoxin and non-endotoxin pyrogens) however, its implementation is considered challenging due to technical challenges for product specific validation. The MAT takes two working days to perform and requires human whole blood (fresh or cryopreserved), peripheral blood mononuclear cells (fresh or cryopreserved) or monocytic cell lines. Kits for performing MAT are available, but it was highlighted during the **Pan-America and Asia regional workshops** that these are considered prohibitively expensive for routine testing. In addition, it was highlighted that it is not easy in all regions to access regular sources of human blood, limiting application of the MAT.

53. The ethical and sustainability issues associated with the LAL/TAL test led to the development of the recombinant factor C (rFC) assay which does not require any animals or animal-derived material. Recombinant factor C is an endotoxin-sensitive synthetic protein that is cloned from factor C DNA, the starting point of the LAL/TAL coagulation cascade. When rFC binds an endotoxin it generates a fluorescent signal that is proportional to the amount of endotoxin present. The rFC assay was first described in 2001 [37] and in a recent review was considered to be "comparable to the more traditional LAL/TAL tests and may be technologically superior" [38].

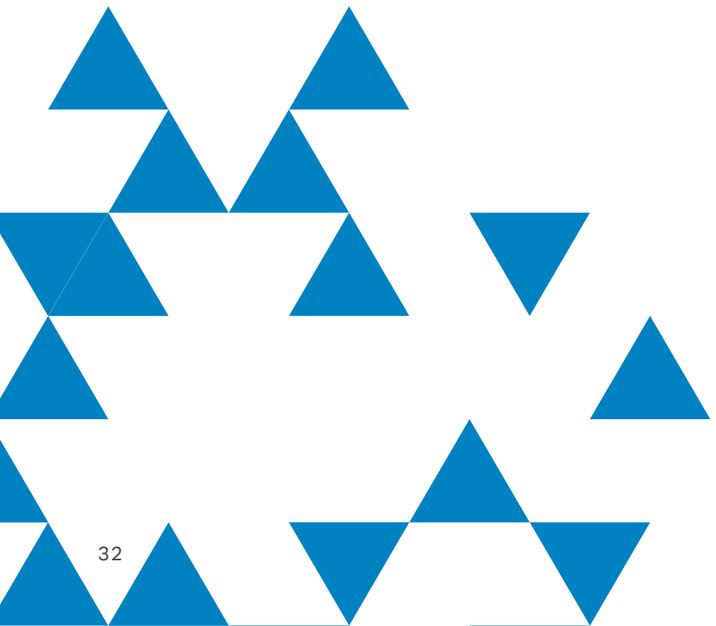
Table 6 – TRS documents recommending animal-based pyrogenicity or endotoxin testing

WHO guideline title	TRS	Year	Product	Page #	Section #
Recommendations for Japanese encephalitis vaccine (inactivated) for human use (revised 2007)	963 Annex 1	2011	Japanese Encephalitis vaccine - (inactivated)	84	A.6.12
Recommendations to assure the quality, safety and efficacy of group A meningococcal conjugate vaccines	962 Annex 2	2011	Meningococcal A conjugate vaccines	126 135	A.3.1.5.6 A.3.6.7
Requirements for meningococcal polysaccharide vaccine	594 Annex 2 658 Annex 6 904 Annex 2	1975 1980 1999	Meningococcal polysaccharides vaccines (unconjugated)	61	A.5.5.1
Recommendations to assure the quality, safety and efficacy of typhoid conjugate vaccines	1030 Annex 2	2021	Typhoid conjugate vaccine	19 22 26	A.4.1.3.9 A.4.4.2 A.6.2.8
Recommendations to assure the quality, safety and efficacy of pneumococcal conjugate vaccines	977 Annex 3	2013	Pneumococcal conjugate vaccines	103	A.3.1.6.6
Recommendations to assure the quality, safety and efficacy of pneumococcal conjugate vaccines	977 Annex 3	2013	Pneumococcal conjugate vaccines	111	A.3.3.10
Recommendations for the production and control of haemophilus influenzae type b conjugate vaccines	897 Annex 1	2000	Haemophilus influenzae type b conjugate vaccines	38 41 45	A3.1.6 A.3.2.2 A.3.6.5
Recommendations for the production and control of meningococcal group C conjugate vaccines (+ addendum 2003)	924 Annex 2 926 Annex 3	2001 2003	Meningococcal group C conjugate vaccines	112 117	A.3.1.6.7 A.3.6.5

WHO guideline title	TRS	Year	Product	Page #	Section #
Requirements for Vi polysaccharide typhoid vaccine	840 Annex 1	1994	Typhoid vaccine polysaccharide	23	A.5.4
Recommendations for whole-cell pertussis vaccine	941 Annex 6	2007	Pertussis vaccine whole cell	314	A.3.3.6
Recommendations to assure the quality, safety and efficacy of acellular pertussis vaccines	979 Annex 4	2013	Pertussis acellular vaccines	199 201	A.3.2.3.1 A.3.3
Manual for Quality Control of Diphtheria, Tetanus and Pertussis Vaccines	WHO/ IVB/ 11.11	2013	DTP vaccines	212	IV.2.2.5
Guidelines on the quality, safety and efficacy of Ebola vaccines	1011 Annex 2	2018	Ebola vaccines	124	A.5.8
Recommendations for the production and control of influenza vaccine (inactivated)	927 Annex 3 1007 Annex 8	2005 2017	Influenza vaccines (inactivated)	121	A.5.5
Recommendations to assure the quality, safety and efficacy of influenza vaccines (human, live attenuated) for intranasal administration	977 Annex 4	2013	Influenza vaccines (live attenuated)	191	A.7.4
Recommendations for inactivated rabies vaccine for human use produced in cell substrates and embryonated eggs	941 Annex 2	2007	Rabies Vaccine	110	A.6.8

WHO guideline title	TRS	Year	Product	Page #	Section #
Guidelines on the quality, safety and efficacy of respiratory syncytial virus vaccines	1024 Annex 2	2020	Respiratory syncytial virus vaccines	146	A.8.10
Recommendations for the production and quality control of smallpox vaccine (revised 2003)	926 Annex 1	2004	Smallpox vaccine	57	A.5.6
Requirements for hepatitis A vaccine (inactivated)	858 Annex 2	1995	Hepititis A vaccine (inactivated)	48	A.6.5
Recommendations to assure the quality, safety and efficacy of recombinant hepatitis B vaccines	978 Annex 4	2013	Hepititis B vaccine (recombinant)	197 202 205	A.3.2 A.6.1.10 A.9.8
Recommendations to assure the quality, safety and efficacy of poliomyelitis vaccines (inactivated)	993 Annex 3 1024 Annex 3	2015 2020	Poliomyelitis vaccines (inactivated)	106 123	A.3.3 A.6.8
Recommendations to assure the quality, safety and efficacy of live attenuated yellow fever vaccines	978 Annex 5	2013	Yellow fever vaccines	260	A.7.9
Guidelines on the quality, safety and efficacy of dengue tetravalent vaccines (live, attenuated)	979 Annex 2	2013	Dengue fever vaccines	66	A.3.1.5
Haemorrhagic fever with renal syndrome (HFRS) vaccines (inactivated)	848 Annex 2	1994	Haemorrhagic fever vaccines	66	A.6.7
Recommendations to assure the quality, safety and efficacy of recombinant hepatitis E vaccines	1016 Annex 2	2019	Hepititis E vaccines (recombinant)	118 122	A.6.3.2 A.9.7
Guidelines to assure the quality, safety and efficacy of live attenuated rotavirus vaccines (oral)	941 Annex 3	2007	Rotovirus vaccine	153	A.4.3.3.6

WHO guideline title	TRS	Year	Product	Page #	Section #
Recommendations to assure the quality, safety and efficacy of recombinant human papillomavirus virus-like particle vaccines	999 Annex 4	2016	HPV	180	A.9.7
Requrements for tick-borne encephalitis vaccine (inactivated)	889 Annex 2	1999	Tick-borne encephalitis vaccine (inactivated)	58	A.6.5
Guidelines on the quality, safety and efficacy of recombinant malaria vaccines targeting the pre-erythrocytic and blood stages of Plasmodium falciparum	980 Annex 3	2014	Malaria vaccines	159 162 163	A.7.1.10 A.10.7 A.10.11.2
Requirements for human interferons prepared from lymphoblastoid cells	786 Annex 3	1989	Human interferons	85 88 89	8.1 10.2 12.5
Guidelines for the production, control and regulation of snake antivenom immunoglobulins	1004 Annex 5	2017	Snake antivenom immunoglobulins	308	17.1.10



54. Proposed new text:

    The need for pyrogenicity testing should be assessed during the manufacturing development process and be re-evaluated following any significant changes in the production process or relevant reported production inconsistencies that may influence pyrogenicity. A risk-based approach should be implemented which is suitable to the manufacturing process and the product depending on the potential presence of endotoxins and non-endotoxin pyrogens.

    The endotoxin content of the final product should be determined using a suitable in vitro assay, such as the recombinant factor C (rFC) or limulus/tachypleus amoebocyte lysate (LAL/TAL) tests. The rFC method is strongly recommended due to concerns over the impact on the sustainability of limulus stocks. The endotoxin content should be consistent with levels found to be acceptable in final product lots used in clinical trials and within the limits agreed upon with the NRA.

    A monocyte activation test (MAT) may be used for pyrogen testing after a product-specific validation. The use of the rabbit pyrogen test should be avoided due to its inherent variability, high retesting rates, and interspecies differences in pyrogenic responses as compared to humans.

55. The focus group recognised that the MAT may be considered a universal pyrogen test but decided it would be more pragmatic, given the technical challenges associated with it, to recommend a 'tiered' approach. In this scenario a bacterial endotoxin test (BET) would be used in preference to a MAT where the risk of non-endotoxin pyrogens has been evaluated during preclinical product and manufacturing process development. The focus group has indicated that the rFC test should be used in preference to the LAL/TAL test due to the ethical concerns around horseshoe crab welfare and preservation.

56. The European pharmacopoeia have signalled its intention to remove RPT from the testing requirements by 2026 [39], while the Indian and the Chinese pharmacopeia have introduce MAT as alternative method to the RPT in 2018 and 2020 respectively. The Brazilian pharmacopeia has recently started to work on the introduction of MAT (2022) after the method has been officially recognised as replacement to RPT in 2019 [40]. Other countries/regions are exploring opportunities to do the same. The focus group recommends that ECBS also considers removal of the RPT from current TRS documents.

57. The focus group recommends that ECBS also considers producing a stand-alone guidance document on pyrogen and endotoxin testing and that a drafting group be established to support this, and which can be referenced in all TRS documents where such testing is currently included. To facilitate this, the focus group has produced a brief outline of material that it believes will be useful in such a guidance document - this is included in Annex 6.

Potency and immunogenicity

58. As with the neurovirulence category, the work of the potency and immunogenicity testing focus group was more complicated because potency/immunogenicity testing is highly product specific and determined during preclinical product and manufacturing process development of the product. Despite this, the focus group felt strongly that a general statement was required, to set out some fundamental principles regarding potency and immungenicity methods used for batch release and quality control purposes. It is proposed that this text should be included in the 'general considerations' section of each relevant TRS document:

    Although animal models are commonly used to determine vaccine potency, animal models have certain shortcomings for use in a control strategy based on consistency. For many vaccines, consistency of production and the monitoring of critical quality attributes using validated physicochemical, biological, or biochemical analytical methods provides a more effective means for quality control. These non-animal methods can be more scientifically relevant than existing animal models because they are more precise, more reproducible, and better suited to vaccine lot release purposes in monitoring those quality attributes which are relevant to its potency.

    If an in vivo test is scientifically justified and required, a risk analysis-based approach should be taken to determine during which production stage (final bulk or final lot) the test should be conducted. In vivo potency testing should not be conducted on both production stages.

59. This statement echoes the principles mentioned previously around determining the appropriate testing strategy during product development using a risk-based approach. The focus group felt strongly that the statement should emphasise the increasingly prevalent view that non-animal testing methods are generally superior to animal-based methods for routine quality control, batch release testing.

60. The focus group reviewed all potency and immunogenicity tests currently described in TRS documents and further sub-categorised these into the following broad categories based on the nature of the test:

    - Unspecified.
    - Challenge test.
    - Serological test with antibody *in vitro* measurement of immune response.
    - Serological test with antibody *in vivo* measurement of immune response.
    - Serological test with unspecified method used for measurement of immune response.

61. The focus group drafted alternative text for products within each of these categories. The output of this is summarised in Table 7.

Table 7 – General text proposed for sub-categories of *in vivo* potency

General text proposed	
Unspecified test	
Viral – RSV, HAV	*As suitable in vitro assays (e.g. ELISA) have been developed and are considered to be appropriate as a potency assay, a quantitative in vitro test, approved by the NRA or using appropriately characterized monoclonal antibodies or other affinity binders, should be performed on each final vaccine bulk or final lot.* *In vitro potency assays are preferred. However, an in vivo assay may be used if scientifically justified and approved by the NRA. Refs: [41, 42]*
Challenge test	
Anti-snake venom Ig	*The use of animals for potency testing of antivenoms raises important ethical considerations and it is essential that 3Rs principles are applied, including use of appropriate analgesia [43], anaesthesia [44], humane endpoints [45], high standards of animal housing, husbandry and care [46] and optimization of experimental design [47] to use the minimum number of experimental animals to measure the potency of an antivenom.* *In this context, developments of appropriate in vitro immunochemical methods validated for replacing animal experiments are strongly encouraged. If an in vitro assay has been developed, it should be implemented as the potency test if approved by the NRA. Refs: [48]*
Bacterial – Whole cell pertussis	*Alternatives to the Kendrick test, based on biological assay systems (e.g. humoral antibody response in sera from a suitable species) have been explored. When shown to be more sensitive and reproducible an alternative assay should be used if approved by the NRA.* *If several final lots are issued from one final bulk product, the biological assay should be carried out on the final bulk product and omitted on the final lots.* *After the demonstration of consistency of production by the biological assay/Kendrick test on an appropriate number of final bulk products, a single dilution assay approved by the NRA should be carried out.* *Biological assays are preferred. However, if the Kendrick test is carried out instead of a serological assay, it must be scientifically justified and should be approved by the NRA.* *In the context of a 3Rs strategy, development of a package of appropriate in vitro biochemical methods, validated for the characterization of the drug product, are strongly encouraged. If an in vitro assay has been developed, it should be implemented as the potency test if approved by the NRA. Refs: [49, 50]*

General text proposed	
Bacterial – Diphtheria Acellular pertussis, Tetanus, DTaP/ DTwP	*As biological assays (e.g. humoral antibody response in sera from a suitable species) with the titration of Ab by in vitro methods (in vitro TNT, ELISA, MIT depending on the component tested) and/or physicochemical tests have been developed and are considered to be more precise and reproducible than the challenge test, a biological and/or physicochemical assay should be used if approved by the NRA.* *In some countries the titration of Abs are performed using multiplex immunological methods for combined DTaP vaccines.* *If several final lots are issued from one final bulk product, the potency assay should be carried out on the final bulk product and omitted on the final lots.* *After the demonstration of consistency of production by the biological and/ or physicochemical assay on an appropriate number of final bulk products, a single dilution assay approved by the NRA should be carried out.* *Biological and/or physicochemical assays are preferred. However, if an in vivo challenge test for Diphtheria, Pertussis & Tetanus is carried out, it must be scientifically justified and should be approved by the NRA. A single dilution assay may be implemented for Diphtheria, Pertussis & Tetanus components after demonstration of consistency of production on an appropriate number of final bulk products and should be approved by the NRA.* *In the context of a 3Rs strategy, development of a package of appropriately validated in vitro assays for the characterization of the drug product is strongly encouraged in order to replace animal models. For all components, in vitro antigenicity assays are being developed and may be considered as potency assays once they are appropriately validated. Refs: [51-54]*
Viral – Rabies, TBEV	*Currently, in vitro assays (e.g. ELISA) have been developed or are under development and may be appropriate as a replacement to the in vivo assay for determination of potency. A quantitative in vitro test, approved by the NRA and using well-characterized antibodies, should be performed on each final vaccine lot.* *In vitro potency assays are preferred, however if an in vivo assay is carried out, it must be scientifically justified and should be approved by the NRA. Refs: [55, 56]*
Viral – Haemorrhagic fever	*Development of appropriate in vitro methods validated for replacing animal models is strongly encouraged. If an in vitro assay has been developed, it should be implemented as the potency test if approved by the NRA. However, if an in vivo assay is carried out instead of an in vitro assay, it must be scientifically justified and should be approved by the NRA.* *If several final lots are issued from one final bulk product, the in vivo assay should be carried out on the final bulk product and omitted on the final lots in order to reduce animal use.* *After the demonstration of consistency of production by the in vivo assay on an appropriate number of final bulk products, a single dilution assay approved by the NRA should be carried out.*

General text proposed	
Serological test with antibody *in vitro*	
Bacterial – Diphtheria Pertussis acellular, Tetanus, DTaP	*As biological assays (e.g. humoral antibody response in sera from a suitable species) with the titration of Ab by in vitro methods (in vitro TNT, ELISA, MIT depending on the component tested) and/or physicochemical tests have been developed and are considered to be more precise and reproducible than the challenge test, a biological and/or physicochemical assay should be used if approved by the NRA.* *In some countries the titration of Abs are performed using multiplex immunological methods for combined DTaP vaccines.* *If several final lots are issued from one final bulk product, the assay should be carried out on the final bulk product and omitted on the final lots in order to reduce animal use.* *After the demonstration of consistency of production by the biological assay on an appropriate number of final bulk products, a single dilution assay approved by the NRA should be carried out.* *In the context of a 3Rs strategy, development of a package of appropriately validated in vitro methods for the characterization of the drug product is strongly encouraged to replace animal experiments. For all components in vitro antigenicity assays are being developed and may be considered as potency assays once they are appropriately validated. Refs: [51-53]*
Parasites, Malaria	*Quantitative in vitro assays (e.g. ELISA) have been developed and are considered appropriate for assessing potency during quality control and batch release testing. Therefore, a quantitative in vitro test, approved by the NRA and using appropriately characterized monoclonal antibodies, should be performed using samples representative of each final vaccine lot. If a biological assay (e.g. humoral response in sera from a suitable species) using in vitro methods for the Ab titration is carried out instead of an in vitro assay, it must be scientifically justified and should be approved by the NRA.* *If several final lots are issued from one final bulk product, the serological assay should be carried out on the final bulk product and omitted on the final lots to reduce animal use.* *After the demonstration of consistency of production by the biological assay on an appropriate number of final bulk products, a single dilution assay approved by the NRA should be carried out. Refs: [57]*

General text proposed	
Viral – JEV, Enterovirus 71	*Quantitative in vitro assays (e.g. ELISA) have been developed and are considered appropriate for assessing potency during quality control and batch release testing. Therefore, a quantitative in vitro test, approved by the NRA and using appropriately characterized monoclonal antibodies, should be performed using samples representative of each final vaccine lot. If a biological assay using in vitro methods for the Ab titration is carried out instead of an in vitro assay, it must be scientifically justified and should be approved by the NRA.* *If several final lots are issued from one final bulk product, the biological assay should be carried out on the final bulk product and omitted on the final lots to reduce animal use.* *After the demonstration of consistency of production by the biological assay on an appropriate number of final bulk products, a single dilution assay approved by the NRA should be carried out. Refs: [58-61]*
Viral – HBV, IPV, HEV, HPV	*In vitro assays for antigen detection (e.g. ELISA) have been developed and are considered to be appropriate for the potency assay. A quantitative in vitro test, approved by the NRA and using appropriately characterized antibodies, should be performed using samples representative of each final vaccine bulk or final lot.* *If an in vivo assay is carried out instead of an in vitro assay, it must be scientifically justified and should be approved by the NRA. Refs: [62-64]*
Serological test with antibody *in vivo*	
Bacterial – Diphtheria Pertussis acellular, Tetanus, DTaP	*As biological assays (e.g. humoral antibody response in sera from a suitable species) with the titration of Ab by in vitro methods (in vitro TNT, ELISA, MIT depending on the component tested) and/or physicochemical tests have been developed and are considered to be more precise and reproducible than the challenge test, a biological and/or physicochemical assay should be used if approved by the NRA.* *In some countries the titration of Abs are performed using multiplex immunological methods for combined DTaP vaccines.* *If several final lots are issued from one final bulk product, the biological assay should be carried out on the final bulk product and omitted on the final lots to reduce animal use.* *After the demonstration of consistency of production by the biological assay on an appropriate number of final bulk products, a single dilution assay approved by the NRA should be carried out.* *In the context of the 3Rs strategy, developments of a package of appropriate in vitro methods validated for the characterization of the drug product are strongly encouraged to replace animal experiments.* *For all components, in vitro antigenicity assays are being developed and may be considered as potency assays once they are appropriately validated. Refs: [52, 53]*

General text proposed	
Serological test with unspecified antibody method	
Synthetic peptides	*In vitro assays, such as monoclonal antibody ELISAs, are likely to be suitable for the routine testing of synthetic peptide vaccines.*
Viral – Rift Valley, Fever, Enterovirus 71	*Quantitative in vitro assays (e.g. ELISA) have been developed and are considered appropriate for assessing potency during quality control and batch release testing. Therefore, a quantitative in vitro test, approved by the NRA and using appropriately characterized monoclonal antibodies, should be performed using samples representative of each final vaccine lot.* *If a biological assay using in vitro methods for the Ab titration is carried out instead of an in vitro assay, it must be scientifically justified and should be approved by the NRA.* *If several final lots are issued from one final bulk product, the serological assay should be carried out on the final bulk product and omitted on the final lots to reduce animal use.* *After the demonstration of consistency of production by the multidose serological assay on an appropriate number of final bulk products, a single dilution assay approved by the NRA should be carried out. Refs: [58]*

62. The proposed alternative text above distils the principles the focus group decided to apply to each of the sub-categories above. It is not intended that this text should, in all cases, replace the current text word-for-word, rather that the principles outlined above should be incorporated when new text is drafted.

Specific toxicity

63. Specific toxicity testing is also highly product specific and consequently, the focus group decided to draft specific revised text for each product. A summary of the original and revised text is provided in Table 8 below. Where the focus group has made a comment about the original text instead of, or as well as proposing new text, the comments are in **bold**.

Table 8 – General text proposed for specific toxicity tests

Product, TRS, section and test name	Original Text	Proposed New Text
BCG vaccine 979 Annex 3 A.3.2.2 Delayed hypersensitivity test	When a new working seed lot is established, a suitable test for delayed hypersensitivity in guinea-pigs is carried out; the vaccine is shown to be not significantly different in activity from the in-house reference.	**Propose no change here. Impact will be low because this test should be used infrequently.**
BCG vaccine 979 Annex 3 A.3.2.6 Test for excessive dermal reactivity	The test for excessive dermal reactivity, described in Part A, section 6.4.2, should be made in six healthy guinea-pigs, each weighing not less than 250 g and having received no treatment likely to interfere with the test. Each guinea-pig should be injected intradermally, according to a randomized plan, with 0.1 ml of the reconstituted vaccine and of vaccine dilutions 1:10 and 1:100. The same dilutions of the appropriate international Reference Reagent or in-house reference should be injected into the same guinea-pigs at randomly selected sites. The guinea-pigs should be observed for at least four weeks. The vaccine complies with the test if the reactions it produces at the sites of injection are not markedly different from those produced by the appropriate international Reference Reagent or in-house reference.	A test for excessive dermal reactivity should be performed. Where available and appropriately validated, an *in vitro* test should be used. If *in vitro* assays are scientifically justified as unavailable and inappropriate, a suitable compendial *in vivo* test may be used.
BCG vaccine 979 Annex 3 A.4.2.3 Test for absence of virulent mycobacteria	At least six healthy guinea-pigs, all of the same sex, each weighing 250–400 g should be used. They should not have received any treatment or diet, such as antibiotics, that is likely to interfere with the test. A sample of the final bulk intended for this test should be stored at 4 °C for not more than 72 hours after harvest. A dose of BCG organisms corresponding to at least 50 single human doses of vaccine intended for intradermal injection should be injected into each guinea-pig by the subcutaneous or intramuscular route.1 The guinea-pigs should be observed for at least six weeks. If, during that time, they remain healthy, gain weight, show no signs of progressive TB and not more than one dies, the final bulk should be considered to be free from virulent mycobacteria.	A test for the absence of virulent mycobacteria should be performed. Where available and appropriately validated, an *in vitro* test should be used (for example a validated nucleic acid amplification test or cell culture method). If *in vitro* assays are scientifically justified as unavailable and inappropriate, a suitable compendial *in vivo* test may be used.

Product, TRS, section and test name	Original Text	Proposed New Text
BCG vaccine 979 Annex 3 A.6.4.1 Test for absence of virulent mycobacteria	Provided the test for virulent mycobacteria has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot. If the test for the absence of virulent mycobacteria, applied to the final bulk, is unsatisfactory (and freedom from progressive TB disease is verified), it should be repeated with a sample of a final lot (see Part A, section 4.2.3).	A test for the absence of virulent mycobacteria should be performed. Where available and appropriately validated, an *in vitro* test should be used (for example a validated nucleic acid amplification test or cell culture method). If *in vitro* assays are scientifically justified as unavailable and inappropriate, a suitable *in vivo* test may be used.
BCG vaccine 979 Annex 3 A.6.4.2 Test for excessive dermal reactivity	Provided the test has been carried out with satisfactory results on the working seed lot and on at least three consecutive final lots produced from it, the test may be omitted on the final lot.	**Keep text**
Diphtheria vaccines 980 Annex 4 General considerations N/A	The assay to detect diphtheria toxin as part of in-process safety testing can be performed using guinea-pigs or using an *in vitro* cell culture system. The purpose of the potency test is to demonstrate, using a suitable animal model, the capacity of the product being tested to induce an immune response analogous to that of toxoid shown to be efficacious in humans.	The assay to detect diphtheria toxin as part of in-process safety testing can be performed using a suitable assay in agreement with the NRA. The Vero cell assay is highly sensitive and is considered superior to existing *in vivo* test methods.
Diphtheria vaccines 980 Annex 4 A.3.3.4 Detoxification and purification	Harvests should be treated as potentially toxic, and subject to the appropriate safety restrictions until the detoxification has been shown to be complete by performance of a specific toxicity test (as detailed in section A.3.4.4) or any other suitably validated *in vivo* or *in vitro* method. SMALL PRINT: Detoxification can be confirmed by subcutaneous inoculation of the toxin into guinea-pigs, or by intradermal injection into guinea-pigs or rabbits. A cell culture assay, such as the Vero cell assay, is also suitable.	Detoxification should be confirmed using a suitable assay in agreement with the NRA. The Vero cell assay is highly sensitive and is considered superior to existing *in vivo* test methods. Once validated, *in vitro* assays should be used for batch release testing.
Diphtheria vaccines 980 Annex 4 A.3.4.4 Specific toxicity	Each bulk purified toxoid should be tested for the presence of diphtheria toxin. The test may be performed *in vivo* using guinea-pigs or *in vitro* using a suitable cell culture assay, such as the Vero cell assay. Some manufacturers carry out an alternative test for determining whether diphtheria toxin is present: they inject intradermally into rabbits or guinea-pigs at least 20 Lf of purified toxoid and observe the injection sites for specific erythema. Erythema with a diameter greater than 5 mm is typically considered to be positive.	The absence of diphtheria toxin should be confirmed using a suitable assay in agreement with the NRA. The Vero cell assay is highly sensitive and is considered superior to existing *in vivo* test methods. Once validated, *in vitro* assays should be used for batch release testing.

Product, TRS, section and test name	Original Text	Proposed New Text
Diphtheria vaccines 980 Annex 4 A.3.4.5 Reversion to toxicity	Each bulk purified toxoid should be tested to ensure that reversion to toxicity does not take place during storage. The test may be performed *in vivo* using guinea pigs or *in vitro* using a suitable cell culture assay, such as the Vero cell assay. The test employed should be approved by the NRA and should be sufficiently sensitive to detect very small amounts of toxin. For the *in vivo* assay, the bulk purified toxoid should be diluted in order to obtain the same concentration and chemical environment as present in the final bulk vaccine SMALL PRINT: For bulk toxoid that will be used in the preparation of more than one final-bulk formulation, the test should be performed using dilutions of the bulk toxoid that represent the lowest and highest concentrations of toxoid that will be present in the final formulations.	Each bulk purified toxoid should be tested to ensure that reversion to toxicity does not take place during storage. The test may be performed using a suitable cell culture assay, such as the Vero cell assay. The test employed should be approved by the NRA and should be sufficiently sensitive to detect very small amounts of toxin.
Diphtheria vaccines 980 Annex 4 A.3.5.2.5 Specific Toxicity	Each final bulk should be tested for specific toxicity in at least five guinea-pigs; each guinea-pig should weigh 250–350 g and not have been used previously for experimental purposes. Each guinea-pig is given a subcutaneous injection of a quantity equivalent to at least 5 SHDs, and is observed for 42 days.	**Remove test. Test is redundant because a more sensitive toxicity test would have already been performed on the bulk purified toxoid at an earlier production stage.**
Diphtheria vaccines 980 Annex 4 A.10.1 Stability	The vaccine should be manufactured in such a way that reversion to toxicity does not occur during the defined shelf-life, provided that the vaccine is stored under the conditions recommended on the label. To confirm that the vaccine does not revert to toxicity during storage, the specific toxicity test described in Part A, section A.3.5.2.5, should be scheduled up until the expiry date as part of the stability studies. In addition, at the time of the expiry date, the vaccine should meet the requirements or acceptance limits for the final product in terms of sterility, potency.	The vaccine should be manufactured in such a way that reversion to toxicity does not occur during the defined shelf-life, provided that the vaccine is stored under the conditions recommended on the label. To confirm that the vaccine does not revert to toxicity during storage, a Vero cell assay can be performed on the final purified bulk at time of the expiry date. If the production method removes the risk of reversion or historical data demonstrate a lack of reversion, this test is not required and should be omitted.
DT-Combined vaccines 980 Annex 6 A.3.2.5 Safety-related testing of aP components (residual activity of pertussis toxin and reversion to toxicity)	In the presence of aluminium-based adjuvants, the *in vitro* Chinese hamster ovary (CHO) cell-based assay may not be applicable for testing the formulated product and for some chemically detoxified antigens. In addition, the *in vivo* test may be sensitive to other components in the formulation rather than to any residual native pertussis toxin (PT) (e.g. aluminium-based adjuvants or IPV). Proper standardization of the *in vivo* test, and the development and introduction of alternative test methods, are strongly encouraged.	The PTx inactivation process should be controlled and demonstrated to consistently reduce the active PTx to levels which are found to be safe in clinical trials. Inactivation of PTx can be monitored using a CHO cell clustering assay or similar *in vitro* method. The CHO cell clustering response has been demonstrated to have greater sensitivity and lower variability than the mouse histamine sensitization test (HIST) and this test is no longer recommended. Although a modified CHO cell clustering method can be used to monitor residual PTx activity in the presence of an adjuvant, inactivation of PTx should be controlled and verified prior to adsorption. The PTx inactivation process should be demonstrated during process development to produce a stable toxoid which does not undergo reversion during downstream processing steps or under recommended storage conditions. Subsequent testing for reversion of PTx should not be necessary once stability of the PTd has been demonstrated. The testing for residual PTx activity or activity from reversion is not necessary for PTd derived from genetic inactivation. The genetic insert should be confirmed to be stable, and the cell line shown to be absent of an active PTx gene.

Product, TRS, section and test name	Original Text	Proposed New Text
DT-Combined vaccines 980 Annex 6 Appendix 1 Absence of residual activity of pertussis toxin	Specify the number, strain and sex of animals used – this test is not necessary for a product obtained by genetic modification.	**Should be removed/revised based on part A text.**
DT-Combined vaccines 980 Annex 6 Appendix 1 Reversion to toxicity of pertussis toxin	Specify the dates of the beginning and end of incubation, and the number, strain and sex of animals used – this test is not necessary for a product obtained by genetic modification.	**Should be removed/revised based on part A text.**
DT-Combined vaccines 980 Annex 6 Appendix 1a Reversion to toxicity	Specify the dates of the beginning and end of incubation, the dates of the beginning and end of the test, the number of animals used, the volume inoculated into cell culture (for diphtheria only) or injected into animals, the number of animals used (if relevant), and the test results.	**Should be removed/revised based on part A text.**
DTP vaccines WHO/IVB/11.11 II.2.1 *In vivo* test for absence of toxin and reversion to toxicity in guinea pigs	The purpose of the specific toxicity test for diphtheria toxin is to confirm freedom from residual toxin and reversion to toxicity in final bulk vaccines and/or bulk purified toxoid. The *in vivo* assay remains the method of choice for routine testing or validation of production processes. The toxicity reversal test for diphtheria toxin is also suitable for the assessment of concentrated toxoid intermediate and is based on the measurement of specific toxicity following incubation of the test toxoid for a prolonged period of time at high temperature to ensure that no reversion of toxoid to toxin has occurred. The WHO specifies the use of the specific toxicity test for the control of purified toxoid bulk and final bulk vaccine, whereas the toxicity reversal assay is only used for the control of purified toxoid bulk [1]. The *in vivo* tests for specific toxicity and toxicity reversal are usually performed in guinea pigs by subcutaneous injection. However, the induction of specific erythema following intradermal injections of at least 20 Lf of purified toxoid can also be used in rabbits and guinea pigs. [1]. Adult, guinea pigs of either sex, each weighing approximately 250-350 g that have not been used for any other test are suitable. Groups of 5 guinea pigs are used per test sample. Ideally, the animals should be acclimatised for a week before starting the test.	**Manual should be revisited following revision of guidelines.**
DTP vaccines WHO/IVB/11.11 II.2.2 Vero cell test for absence of toxin and reversion to toxicity	A Vero cell culture system may be used as an alternative to *in vivo* tests for specific toxicity and reversion to toxicity as long as sensitivity of the assay is shown to be comparable to the guinea pig test [1]. WHO recommends the use of Vero cell culture assay provided that the test is validated against the guinea pig test. During such validation studies recommendations regarding pass/fail requirements can be made.	**Manual should be revisited following revision of guidelines.**

Product, TRS, section and test name	Original Text	Proposed New Text
DTP vaccines WHO/IVB/11.11 III.2.1 *In vivo* test for absence of toxin and reversion to toxicity in guinea pigs	The purpose of the specific toxicity test for tetanus is to confirm freedom from residual toxin and reversion to toxicity in final bulk vaccines and/or bulk purified toxoid. The toxicity reversal test for tetanus is also suitable for the assessment of concentrated toxoid intermediate product, and is based on the measurement of specific toxicity following incubation of the test toxoid for a prolonged period of time at high temperature to ensure that no reversion of toxoid to toxin has occurred. The tests for specific toxicity and toxicity reversal are usually performed in guinea pigs by subcutaneous injection. Although mice are not as sensitive to tetanus toxin as guinea pigs, WHO allows the use of mice for the toxicity reversal test, subject to approval by the National Regulatory Authority.	**Manual should be revisited following revision of guidelines.**
DTP vaccines WHO/IVB/11.11 IV.2.1 Mouse weight gain test (MWGT)	The MWGT is considered as a general, non-specific test measuring overall toxicity of pertussis whole cell vaccine, since a number of B. pertussis toxins may induce weight loss in mice. Correlation of the results of the MWGT with adverse reactions in children has been reported [3-6]. It is a test used to assess the toxicity of whole cell pertussis containing vaccines, and it is based on the ability of certain toxins or components from B. pertussis to cause weight loss in young mice.	**Manual should be revisited following revision of guidelines.**
DTP vaccines WHO/IVB/11.11 IV.2.2.2.1 Histamine sensitization assay (Temperature method)	Mice inoculated with pertussis toxin become highly sensitive to a histamine challenge. The effects include reduction in body temperature and in the severe cases death. The reduction in body temperature occurs within 30 minutes after histamine challenge, but in the non-lethal situations it returns to normal levels after 30 minutes. Therefore, reduction in body temperature 30 minutes following histamine challenge is directly proportional to the dose of active PT present in the vaccine. This method is highly sensitive, it can detect levels of PT activity that do not induce lethal effects following histamine challenge. Body temperature in mice can be assessed by measuring rectal or dermal temperature using either an electric thermometer with a probe specific for mice or an infrared thermometer, respectively. Results are obtained as continuous variables so as to allow calculating mean and variance for each group. Rectal temperature method has been used in Japan since 1981 [2, 3]. Assessment of body temperature by both methods (rectal and dermal) correlates with PT toxicity in animals. However, for practical reasons dermal measurements are preferred in some countries.	**Manual should be revisited following revision of guidelines.**

Product, TRS, section and test name	Original Text	Proposed New Text
DTP vaccines WHO/IVB/11.11 IV.2.2.2.2 Histamine sensitization assay (Lethal end-point method)	An assay to assess the active pertussis toxin (PT) content of pertussis containing vaccines on the basis of the histamine sensitising effect of active PT on mice. Pertussis toxin increases the sensitivity of mice to histamine. The exact mode of action is not yet fully understood. Even when small amounts of active PT are present in a vaccine, mice will become vulnerable to challenge with histamine, resulting in anaphylactic shock and inevitable death. The amount of histamine sensitisation factor (HSF) activity in a vaccine can be quantified in a parallel-line assay in comparison with a reference vaccine. In this assay the reference and test vaccine doses, which induce a histamine sensitisation in 50% of the animals, as measured by death after challenge with histamine, are compared and a relative HSF activity is calculated for the vaccine. Different mouse strains may show different sensitivity to the test, laboratories are recommended to set up their own experimental conditions.	**Manual should be revisited following revision of guidelines.**
DTP vaccines WHO/IVB/11.11 IV.2.2.3 Heat-Labile Toxin (HLT) test (Dermonecrotic toxin test)	Heat-labile toxin (HLT) is a heat labile protein toxin of B. pertussis which can be inactivated in 10 minutes at 56°C. HLT is dermonecrotizing, lethal, and causes spleen atrophy in experimental animals. Since its discovery by Bordet and Gengou (1909), the toxin has been considered to play an important role in pathogenicity, most probably in the initial stage of whooping cough. The toxin is produced by all phase I B. pertussis strains. Pertussis vaccine should not contain biologically active HLT. Absence of HLT is not considered to be a product release criterion but validation of the manufacturing process should demonstrate the absence of HLT in the pertussis bulk after inactivation. Suckling mice are most responsive to the lethal or dermonecrotizing activity of HLT after subcutaneous injection into the nuchal area. Both lethality and dermonecrocity, can be used as parameters. As a negative control saline may be used or heat inactivated sample (56°C, 10 minutes). Usually one to three dilutions per sample are tested.	**Manual should be revisited following revision of guidelines.**
DTP vaccines WHO/IVB/11.11 IV.2.2.4 Leukocytosis Promotion (LP) Test	Pertussis vaccine contains pertussis toxin, formerly Leukocytosis Promoting Factor (LPF) that enhances the number of circulating leukocytes. Although most methods applied for determining the pertussis toxin-induced leukocytosis do not discriminate between leukocytes and lymphocytes some people persist in calling it lymphocytosis promoting factor. The LP activity of a pertussis vaccine is estimated by counting the number of circulating leukocytes 7 days after injection of mice with the test vaccine. Counting the number of circulating leukocytes can be done either by haemocytometer or electronic cell counter. In some laboratories, this is done in combination with MWGT.	**Manual should be revisited following revision of guidelines.**

Product, TRS, section and test name	Original Text	Proposed New Text
Haemophilus influenzae type b conjugate vaccines 897 Annex 1 A.3.3.10 Specific toxicity of carrier protein in the conjugate	The bulk conjugate should be tested for the absence of specific toxicity of the carrier protein where appropriate. Absence of specific toxicity of the carrier protein may also be assessed through validation of the production process.	**Recommend that this test is moved to the 'Control of the carrier protein' section.** The carrier protein should be tested for the absence of specific toxicity of the carrier protein where appropriate (e.g. when tetanus or diphtheria toxoids have been used). Absence of specific toxicity of the carrier protein may also be assessed through validation of the production process. Where available and appropriately validated and with approval from the NRA, an *in vitro* test should be used. If *in vitro* assays are scientifically justified as unavailable and inappropriate, a suitable *in vivo* test may be used.
Haemorrhagic fever vaccines 848 Annex 2 A.4.4.2 Inactivation of virus	Option (B) - Each of at least 10 mice of two days old is inoculated intracerebrally. Option (C) - Suckling mice and hamsters are inoculated with culture fluid.	Each undiluted bulk suspension shall be tested for inactivation of the virus by an *in vitro* method approved by the NRA (e.g. immunoassay for hantaviral antigen following inoculation of test sample into a suitable cell culture). If an *in vivo* inactivation assay is conducted, it should be specifically justified and approved by the NRA.
Haemorrhagic fever vaccines 848 Annex 2 A.6.11 Test for residual live virus	In some countries. Inoculating 10 mice intracerebrally.	**This can be deleted in light of recommended change to section A.4.4.2.**
Meningococcal A conjugate vaccines 962 Annex 2 A.3.3.10 Specific toxicity of carrier protein	The bulk conjugate should be tested for the absence of specific toxicity of the carrier protein where appropriate (when tetanus or diphtheria toxoids have been used). Absence of specific toxicity of the carrier protein may also be assessed through validation of the production process.	**Recommend that this test is moved to the 'Control of the carrier protein'section.** The carrier protein should be tested for the absence of specific toxicity of the carrier protein where appropriate (e.g. when tetanus or diphtheria toxoids have been used). Absence of specific toxicity of the carrier protein may also be assessed through validation of the production process. Where available and appropriately validated and with approval from the NRA, an *in vitro* test should be used. If *in vitro* assays are scientifically justified as unavailable and inappropriate, a suitable *in vivo* test may be used.
Meningococcal group C conjugate vaccines 924 Annex 2 926 Annex 3 A.3.3.10 Specific toxicity of carrier protein	The bulk conjugate should be tested for the absence of specific toxicity of the carrier protein where appropriate (e.g. when tetanus or diphtheria toxoids have been used). Absence of specific toxicity of the carrier protein may also be assessed through validation of the production process.	**Recommend that this test is moved to the 'Control of the carrier protein' section.** The carrier protein should be tested for the absence of specific toxicity of the carrier protein where appropriate (e.g. when tetanus or diphtheria toxoids have been used). Absence of specific toxicity of the carrier protein may also be assessed through validation of the production process. Where available and appropriately validated and with approval from the NRA, an *in vitro* test should be used. If *in vitro* assays are scientifically justified as unavailable and inappropriate, a suitable *in vivo* test may be used.

Product, TRS, section and test name	Original Text	Proposed New Text
MMR 840 Annex 3 Appendix 2 Freedom from tumorigenicity	The cells of the MWCB shall be shown to be free from potential tumorigenicity by appropriate animal tests, including positive controls, approved by the national control authority. Suitable tests in immunosuppressed animals are as follows. Approximately 10^6 cells obtained from cultures at the same passage levels as those to be used for vaccine production are injected into: new-born mice or hamsters treated with antilymphocyte serum; or athymic mice (nude nulnu genotype); or thymectomized, irradiated mice with reconstituted bone marrow (T-B+). Some of the same group of animals should be inoculated with a similar dose of HeLa or KB cells as positive controls. The animals should be observed for not less than three weeks. Other tests in animals treated with immunosuppressive agents and with equal sensitivity to neoplastic cells may also be used. The test Is valid if the positive control animals develop tumours. The cells are suitable for vaccine production if at least 80% of inoculated animals remain healthy and survive the observation period, and none of the animals shows evidence of tumour formation attributable to the cells.	**Should be removed/revised based on part A text.**
Oral cholera vaccine 924 Annex 3 General considerations 2.6 Production and control of inactivated oral cholera vaccines	The mouse weight-gain test currently in use to monitor the toxicity of vaccine lots is considered to be insufficiently sensitive and of questionable relevance. A more relevant and validated test should be sought.	The mouse weight-gain test currently in use to monitor the toxicity of vaccine lots is considered to be insufficiently sensitive and of questionable relevance. A more relevant and validated test should be sought. The potential use of the Y-1 adrenal cell assay for cholera toxin as a more specific test for residual toxicity should be investigated. Such a specific test could be used on a-lot-to-lot basis or to validate the production process.
Pertussis acellular vaccines 979 Annex 4 A.3.3 Residual activity of pertussis toxin	The amount of residual biologically active PT in the individually or co-purified antigens should be estimated after detoxification by means of a sufficiently sensitive test such as the HIST or the CHO cell assay.	The PTx inactivation process should be controlled and demonstrated to consistently reduce the active PTx to levels which are found to be safe in clinical trials. Inactivation of PTx can be monitored using a CHO cell clustering assay or similar *in vitro* method. The CHO cell clustering response has been demonstrated to have greater sensitivity and lower variability than the mouse histamine sensitization test (HIST) which is no longer recommended. Although a modified CHO cell clustering method can be used to monitor residual PTx activity in the presence of an adjuvant, inactivation of PTx should be controlled and verified prior to adsorption. The PTx inactivation process should be demonstrated during process development to produce a stable toxoid which does not undergo reversion during downstream processing steps or under recommended storage conditions. Subsequent testing for reversion of PTx should not be necessary once stability of the PTd has been demonstrated. The testing for residual PTx activity or activity from reversion is not necessary for PTd derived from genetic inactivation. The genetic insert should be confirmed to be stable, and the cell line shown to be absent of an active PTx gene.

Product, TRS, section and test name	Original Text	Proposed New Text
Pertussis acellular vaccines 979 Annex 4 A.3.4.2.5 Residual activity of pertussis toxin	Each final bulk of vaccine should be tested for active PT using a HIST or another test that is sufficiently sensitive to detect the level of toxin activity agreed with the NRA.	**This test may not be needed at this stage if the test has been applied at an earlier stage. Otherwise use same text as A.3.3.**
Pertussis acellular vaccines 979 Annex 4 A.3.4.2.6 Reversion to toxicity	Accelerated reversion testing, consisting of HIST performance on final bulk or the final lot incubated for at least four weeks at 37 °C, may be used to demonstrate that it is unlikely that the chemically inactivated PT will regain some of its toxicity before the vaccine expiry date. Some NRAs may not require this test for the release of each new lot but only as part of process validation.	**This test may not be needed at this stage if the test has been applied at an earlier stage. Otherwise use same text as A.3.3.**
Pertussis acellular vaccines 979 Annex 4 Appendix 2 Histamine sensitization test by temperature measurement	Detailed protocol for HIST assay with temperature change.	**Should be removed/revised based on part A text.**
Pertussis acellular vaccines 979 Annex 4 Appendix 3 Histamine sensitization test by lethal end-point assay	Detailed protocol for HIST assay with lethal endpoint.	**Should be removed/revised based on part A text.**
Pertussis vaccine Whole cell 941 Annex 6 A.3.3.6 Specific toxicity	Each final bulk should be tested for toxicity using the mouse weight gain test. The final bulk is considered satisfactory if the following conditions are met: (a) at the end of 72 hours the average weight of the group of vaccinated mice is not less than that preceding the injection, (b) at the end of 7 days the average weight gain per mouse is not less than 60% of that per control mouse, and (c) no deaths occur when 10 mice are used and no more than one death occurs when 20 mice are used.	Each final bulk should be tested for toxicity using a suitable assay. The assay must be sensitive to all potential toxins that may be present. The presence of toxins should be determined during product development. The mouse weight gain test is considered imprecise and in need of replacement. Manufacturers should develop alternatives or refinement of the mouse weight gain test.

Product, TRS, section and test name	Original Text	Proposed New Text
Pertussis vaccine Whole cell 941 Annex 6 A.3.3.6 Specific toxicity Pertussis toxin	A Chinese hamster ovary cell (CHO-cell) assay, based on the clustering of cells after treatment with pertussis toxin is used. Tests for histamine sensitizing activity in mice may also be used.	If required by the NRA, a specific toxicity test may be performed using a suitable, validated cell-based assay (e.g. CHO-cell assay). The assay should be performed using diluted vaccine. A histamine sensitizing activity test in mice may only be used where alternatives are not possible.
Pneumococcal conjugate vaccines 977 Annex 3 A.3.3.9 Specific toxicity of carrier protein	The bulk conjugate should be tested for the absence of specific toxicity of the carrier protein where appropriate (e.g. when tetanus or diphtheria toxoids have been used). Absence of specific toxicity of the carrier protein may also be assessed through validation of the production process.	**Recommend that this test is moved to the 'Control of the carrier protein' section.** The carrier protein should be tested for the absence of specific toxicity of the carrier protein where appropriate (e.g. when tetanus or diphtheria toxoids have been used). Absence of specific toxicity of the carrier protein may also be assessed through validation of the production process. Where available and appropriately validated and with approval from the NRA, an *in vitro* test should be used. If *in vitro* assays are scientifically justified as unavailable and inappropriate, a suitable *in vivo* test may be used.
Pneumococcal conjugate vaccines 977 Annex 3 A.3.3.9 Specific toxicity of carrier protein	The bulk conjugate should be tested for the absence of specific toxicity of the carrier protein where appropriate (e.g. when tetanus or diphtheria toxoids have been used). Absence of specific toxicity of the carrier protein may also be assessed through validation of the production process.	**Recommend that this test is moved to the 'Control of the carrier protein' section.** The carrier protein should be tested for the absence of specific toxicity of the carrier protein where appropriate (e.g. when tetanus or diphtheria toxoids have been used). Absence of specific toxicity of the carrier protein may also be assessed through validation of the production process. Where available and appropriately validated and with approval from the NRA, an *in vitro* test should be used. If *in vitro* assays are scientifically justified as unavailable and inappropriate, a suitable *in vivo* test may be used.
Poliomyelitis vaccines (inactivated) 993 Annex 3 1024 Annex 3 A.4.5.2 Test for effective inactivation	After removal or neutralization of the inactivating agent, the samples should be tested for the absence of infective poliovirus by inoculation into tissue cultures. Kidney cells from some monkey species, such as those of the genera Macaca, Cercopithecus and Papio sp., appear to be more sensitive than others. When primary monkey kidney cells are used for this test, the two samples should be inoculated into culture vessels of tissue cultures derived from different batches of cells.	After removal or neutralization of the inactivating agent, the samples should be tested for the absence of infective poliovirus by inoculation into tissue cultures. Wherever possible, established and validated continuous cell lines should be used in preference over the use of primary cells.
Poliomyelitis vaccines (oral, live, attenuated) 1045 Annex 2 E.1.4.1.1 Tests in rabbits	A sample of the monovalent bulk should be tested for the presence of herpes B virus and other viruses by injection into at least 10 healthy rabbits, each weighing between 1.5 kg and 2.5 kg. All rabbits that die after the first 24 hours of the test should be examined by necropsy, with the brain and organs removed for detailed examination to establish the cause of death. Animals showing signs of illness should be humanely killed and subjected to a similar necropsy. A test for the presence of Marburg virus may be carried out in guinea pigs.	**Text to be added to section E:** **These tests can be avoided if primary monkey kidney cells are not used for vaccine production. OPV manufacturers are encouraged to avoid the use of primary animal cells for vaccine production.** **Where animal tests are still required, the use of humane endpoints are encouraged. Manufacturers are also encouraged to adopt contemporary best practice for animal care and welfare.**

Product, TRS, section and test name	Original Text	Proposed New Text
Rift Valley Fever vaccine 673 Annex 4 3.4.2 Test in rabbits	A sample of at least 30 ml of each single pool shall be tested as soon as possible after pooling by inoculation into three healthy rabbits, each weighing between 1.5 and 2.5 kg; proportionately larger volumes shall be used if more animals are inoculated. The inoculations shall be made at multiple sites, each rabbit being given a total of 1 ml of the single pool by intradermal injection and 9 ml by subcutaneous injection. The animals shall be observed for at least three weeks. All rabbits that die after the first 24 h of the test or that show signs of illness shall be examined by autopsy, with removal of the brain and organs for detailed inspection. The single pool passes the test if at least 2 of the rabbits remain healthy and if none of the rabbits shows lesions of any kind at the sites of inoculation or shows evidence of infection with B virus or with any adventitious transmissible agent attributable to the single pool.	**There is no vaccine for human use, this is for veterinary use only. The whole document may be out of date and needs wholesale revision/removal.**
Tetanus vaccines 980 Annex 5 A.3.3.4 Detoxification and purification	Harvests should be treated as potentially toxic, and subject to the appropriate safety restrictions until the detoxification has been shown to be complete by performance of a specific toxicity test (as detailed in section A.3.4.4) or any other suitable *in vivo* method.	Harvests should be treated as potentially toxic, and subject to the appropriate safety restrictions until the detoxification has been shown to be complete.
Tetanus vaccines 980 Annex 5 A.3.4.4 Specific toxicity	Each bulk purified toxoid, diluted with the same buffer solution as used in the final vaccine, should be tested for the absence of tetanus toxin in guinea-pigs; the guinea-pigs should each weigh 250–350 g and not previously have been used for experimental purposes. At least five guinea-pigs should be injected subcutaneously with 1 ml of a dilution of purified tetanus toxoid containing at least 500 Lf of toxoid; they must be observed daily for signs of tetanic paralysis over a period of 21 days. A suitable method is outlined in the WHO Manual for quality control of diphtheria, tetanus and pertussis vaccines (45). Animals that die, whatever the cause, will need to be examined by necropsy.	Each bulk purified toxoid, diluted with the same buffer solution as used in the final vaccine, should be tested for the absence of tetanus toxin. *In vitro* assays (e.g. BINACLE assay) are being developed and are currently undergoing validation (EDQM BSP 136). Inactivation of tetanus toxin can be monitored using an *in vitro* assay (once approved by the NRA) or a suitable *in vivo* assay. The tetanus toxin inactivation process should be demonstrated during process development to produce a stable toxoid which does not undergo reversion during downstream processing steps or under recommended storage conditions. Subsequent testing for reversion of tetanus toxin should not be necessary once stability of the product has been demonstrated with sufficient historical evidence.
Tetanus vaccines 980 Annex 5 A.3.4.5 Reversion to toxicity	The diluted toxoid sample is incubated at 34–37 °C for a period of six weeks (42 days). At the end of the incubation period, five guinea-pigs are each injected subcutaneously with 5.0 ml (i.e. 10 human doses, using multiple injection sites where necessary) of test sample. The animals are observed for 21 days for signs of ill health. No toxicity should be detected. The bulk purified toxoid passes the test if no guinea-pig shows symptoms of specific paralysis or any other signs of tetanus within 21 days of injection.	**Remove as described, keep requirement to validate lack of reversion during development.**

Product, TRS, section and test name	Original Text	Proposed New Text
Tetanus vaccines 980 Annex 5 A.3.5.2.5 Specific toxicity	Each final bulk should be tested for specific toxicity in at least five guinea-pigs; each guinea-pig should weigh 250–350 g and not have been used previously for experimental purposes. Each guinea-pig is given a subcutaneous injection of a quantity equivalent to at least 5 SHD, and is observed daily for a period of 21 days. Animals that die from any cause should undergo necropsy and be inspected for symptoms of tetanus paralysis.	**This test is redundant and should be removed.**
Tetanus vaccines 980 Annex 5 A.10.1 Stability	To confirm that the vaccine does not revert to toxicity during storage, the specific toxicity test described in Part A, section A.3.5.2.5, should be scheduled up to the expiry date as part of the stability studies. In addition, at the time of the expiry date, the vaccine should meet the requirements for the final product in terms of sterility, potency, adjuvant content, degree of adsorption, preservative content, pH and extractable volume, where applicable (as described in Part A, sections A.5.2, A.5.3 and A.5.5–A.5.9), provided that the vaccine has been stored at the recommended temperature.	A suitable *in vitro* assay (e.g. the BINACLE assay) should be performed on the final purified bulk at the time of the expiry date. If the production method removes the risk of reversion or historical data demonstrate a lack of reversion, the test is not required and could be omitted following discussion and approval from the NRA.
Typhoid conjugate vaccine Tbc A.4.4.13 Specific toxicity of the carrier protein	The purified bulk conjugate should be tested to confirm the absence of toxicity specific to the carrier protein where appropriate (for example, when DT or TT is used as the carrier protein). Alternatively, the absence of specific toxicity of the carrier protein may be demonstrated at the purified carrier protein stage if agreed with the NRA.	**Recommend that this test is moved to the 'Control of the carrier protein' section.** The carrier protein should be tested for the absence of specific toxicity of the carrier protein where appropriate (e.g. when tetanus or diphtheria toxoids have been used). Absence of specific toxicity of the carrier protein may also be assessed through validation of the production process. Where available and appropriately validated and with approval from the NRA, an *in vitro* test should be used. If *in vitro* assays are scientifically justified as unavailable and inappropriate, a suitable *in vivo* test may be used.
Typhoid conjugate vaccine Tbc Appendix Section 2 Specific toxicity of carrier protein (where appropriate)	Method used: Strain and type of animals: Number of animals: Route of injection: Volume of injection: Quantity of protein injected: Date of start of test: Date of end of test: Specification: Result:	**Appendix (lot summary release certificate), should be revised based on changes made in rest of TRS.**

64. Consistent with the work of the other focus groups, the specific toxicity focus group promoted non-animal technology where possible and removed unnecessary detail around animal tests.

Additional output from the review process

Stakeholder engagement

65. There are a number of distinct communities that facilitate quality control, batch release testing of vaccines and biological therapeutics. These include manufacturers, NCLs and NRAs and each of these are likely to have subtle, but important, regional variations in current approaches for or options on implementation of the 3Rs. From the outset of the project, it was clear that regular stakeholder engagement with these communities was essential if the recommendations within this report are to be implemented by WHO and welcomed and accepted by the end-users of WHO guidelines. We undertook both community surveys and regional workshops as complementary stakeholder engagement approaches.

Surveys

66. Two surveys were performed during the project – the first targeted at the manufacturers of vaccines and biological therapeutics and the second targeted at the regulatory community (both NCLs and NRAs).

67. Both surveys were distributed as Microsoft Excel files and consisted of three sections:

    i. Demographic data.

    ii. Questions regarding current practices with respect to animal-based methods for quality control and batch release testing of vaccines and biological therapeutics.

    iii. Questions regarding opportunities and barriers to the adoption of 3Rs and non-animal technology methods used in the quality control and batch release testing of vaccines and biological therapeutics.

68. Prior to distribution, both surveys passed ethical review by the Royal Veterinary College (RVC, London, UK) Social Science Research Ethical Review Board (Manufacturers survey - RVC ref: URN SR2021-0131; Regulatory survey - RVC ref: URN: SR2021-0169). The surveys were distributed (via a link to the NC3Rs website) through advertising on social media, direct email to relevant networks and industry newsletters and websites.

Manufacturers survey

69. The manufacturers survey was launched in July 2021 and formally closed in September 2021 (though a small number of responses were received after this date and were included in the analysis). Thirty complete responses from 25 different vaccine and biological therapeutics manufacturers were received. Multiple submissions were allowed from multi-national manufacturers where the submissions were from distinct subsidiaries or country locations. The nature of the survey dissemination means that we do not know how many individual manufacturers were aware of the survey and therefore, we cannot judge the overall response rate.

70. The survey set out to gain greater understanding on how animals are used by vaccine and biological therapeutics manufacturers in quality control and batch release testing and to explore barriers and opportunities for greater implementation of the 3Rs. Animals are still widely used – 416 individual animal tests spanning 20 different animal-based methods for 154 products were reported by the 30 manufacturers who responded to the survey. Although there was good awareness of 3Rs approaches and non-animal technologies in many test categories, their use was very low overall. The most common test categories where animals are used consistently were potency (29.3% of all animal use) followed by ATT/GST (20.5%), adventitious agents (11.2%), specific toxicity (11%) and pyrogen testing (9.3%). It is particularly interesting that the ATT/GST test is still widely used given that the WHO, FDA and several national pharmacopeia have deleted the requirement for this test from their guidelines. We specifically asked whether respondents were aware that the WHO had deleted the ATT/GST and nearly 80% indicated that they were aware of the decision of ECBS in 2018. While current adoption of non-animal technologies may be low, there is a clear appetite from manufacturers to engage with regulatory authorities on how to transition away from animal tests because they recognise the scientific, commercial and animal welfare benefits this offers. Most manufacturers who completed the survey indicated that they have engaged with regulators on this and that these discussions were positive, although in some cases more data were needed to convince regulators of the validity of the non-animal technologies presented.

71. For more information about the survey and a detailed discussion of the responses see the paper in Annex 3.

Regulatory survey

72. The regulators survey was launched in January 2022 and formally closed in April 2022 (though a small number of responses were received after this date and were included in the analysis). Thirty-four responses were received with 31 completed surveys (two responses came from NRAs indicating that they did not perform any animal testing and one incomplete response was received). The nature of the survey dissemination means that we do not know how many individual NRAs and NCLs were aware of the survey and therefore, we cannot judge the overall response rate.

73. Similar to the manufacturers survey, the regulatory survey set out to gain greater understanding on how animals are used by NCLs for quality control and batch release testing, how regulators regard non-animal technologies and to explore barriers and opportunities for greater implementation of the 3Rs. Completed surveys were received from 29 different countries. Two separate responses (NRA and NCL) were received from both the United Kingdom and the Netherlands. Most responses were from respondents based in Europe, although responses were received from the Americas, Africa and Oceania. In total, there were 29 sets of NCL data and 13 sets of NRA data. Most NRAs indicated that the uptake of non-animal technology was important although most had not actively engaged in discussions with manufacturers to facilitate this. Interestingly, awareness of the WHO decision to no longer require the ATT/GST from NRAs (83%) was similar to that of manufacturers in the previous survey. However, 69% of NRAs still received ATT/GST data in submissions from manufacturers. There was good awareness of the 3Rs from both NRA and NCL respondents with 100% indicating that use of non-animal technologies was important because of concerns around ethics, variability of animal assays and the benefits of reducing quality control time and costs when non-animal test methods are used.

74. For more information about the survey and a detailed discussion of the responses see the paper in Annex 3.

Key messages from both surveys

75. Overall awareness of the 3Rs across manufacturer and regulatory communities was very high reflecting the fact that, following many years of promotion by organisations like the NC3Rs, these principles are now part of the common lexicon of science communication and policy. Although the ethical concerns over animal use are a factor for implementation of the 3Rs, there was good awareness of the clear, robust scientific arguments for transitioning away from animal testing. Respondents indicated that they considered animal tests to be more variable, more expensive and more time consuming to run than *in vitro*-based methods.

76. As well as general questions around the 3Rs, both surveys asked respondents whether they believed that the project objective to revise WHO guidelines and the proposal of the working group for WHO to develop a general 3Rs guideline or position statement, would be beneficial. Overwhelmingly, respondents in both surveys indicated that they supported both of these options.

Regional workshops

77. The NC3Rs hosted a series of regional workshops to better understand the potential impact of any proposed changes to WHO TRS documents on manufacturers and regulators globally. These workshops were hosted virtually in light of the ongoing COVID pandemic and to encourage the widest possible participation from across the regions.

78. Three regional workshops were hosted in 2022:

- Europe: Wednesday 2 March 2022.
- Asia: Thursday 28 April 2022.
- Pan-America: Monday 26 September 2022.

79. Each workshop was organised by a local organising committee and agendas were tailored to be regionally relevant with respect to current knowledge of the 3Rs and the potential impact of proposed changes to WHO TRS documents. The organising committee membership and the final agendas for each of the workshops can be found in Annex 8.

80. In each workshop, participants recognised the benefits of moving to non-animal approaches including lower variability of *in vitro* assays, cost and time savings, reducing animal use and ethical concerns around animal welfare. In Europe, the 3Rs are enshrined in legislation (Directive 2010/63/EU of the European Parliament[2]), enabling widespread adoption of 3Rs approaches within the European Pharmacopeia and in national regulations. In particular, inclusion of chapter 5.2.14 (Substitution of *in vivo* method(s) by *in vitro* method(s) for the quality control of vaccines) in the European Pharmacopeia in 2018 [65] was reported as a significant step in the promotion of alternative methods. The European workshop made particular mention of the VAC2VAC project which brought together scientists from both the human and veterinary pharmaceutical industry along with academia and regulators. The VAC2VAC consortium developed new non-animal tests and generated vaccine specific toolkits for consistency testing[3]. This was a considerable achievement and emphasises the desire that the vaccines and biological therapeutics community has for implementation of the 3Rs.

81. The Asia-region workshop suggested that, whilst the desire to adopt the 3Rs is strong, there is a need for training and support in accessing standards, reagents and expertise to enable uptake of alternative methods. Asian speakers and delegates also indicated that they were strongly influenced by WHO guidelines and expressed enthusiasm for WHO to provide greater integration of 3Rs approaches going forward. There was significant willingness across the region to take part in collaborative studies, for example those run by the Developing Countries Vaccine Manufacturers Network (DCVMN), which was seen as important to facilitate the adoption of non-animal technologies. Access to affordable non-animal methods was indicated as a barrier to adoption with kit-based MAT assays specifically mentioned as being too expensive for routine use.

82. Many of the issues highlighted in the European and Asian workshops were echoed in the Pan-American meeting. There was a clear appetite for the transition towards 3Rs approaches from all of the regions present. However, there were divergent challenges between North America and Central/South America in that some Low- or Middle-Income Countries (LMIC) lacked resources to access certain equipment and reagents necessary for non-animal technologies and were more heavily influenced by other national pharmacopeia and WHO guidelines, taking confidence in adopting non-animal test methods when they are adopted in these resources.

83. All three workshops supported the aims of this project and welcomed both wider integration of 3Rs methods in WHO guidelines as well as WHO guidance on implementing the 3Rs (see Annex 7).

[2] eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:276:0033:0079:en:PDF

[3] www.imi.europa.eu/projects-results/project-factsheets/vac2vac

Observations on WHO ECBS processes

84. The **project scope** was intended to focus the rationale for this project and the recommendations around 3Rs principles on scientific evidence and to avoid any non-constructive criticism of the WHO. During the review, several observations were made regarding WHO processes that may benefit from some refinements. This section of the report describes these observations and includes suggestions for changes in current practice that could improve access and utility of WHO guidance documents going forward. In keeping with the original scope, this is not intended as criticism of WHO.

85. This project principally involved the review of WHO TRS documents that are publicly available on the WHO website. These documents (published on the WHO Biologicals web pages as PDF files[4]) are contained as annexes to the reports of ECBS meetings and which are formatted for printing as physical media ('TRS books'). The review process highlighted some significant challenges with accessing and working with the TRS documents. The main issue was version control – some product guidance consisted of an original document and multiple amendments all of which were published as annexes in separate ECBS meeting reports. One example of this is the Meningococcal polysaccharide vaccine guideline which is spread over three documents:

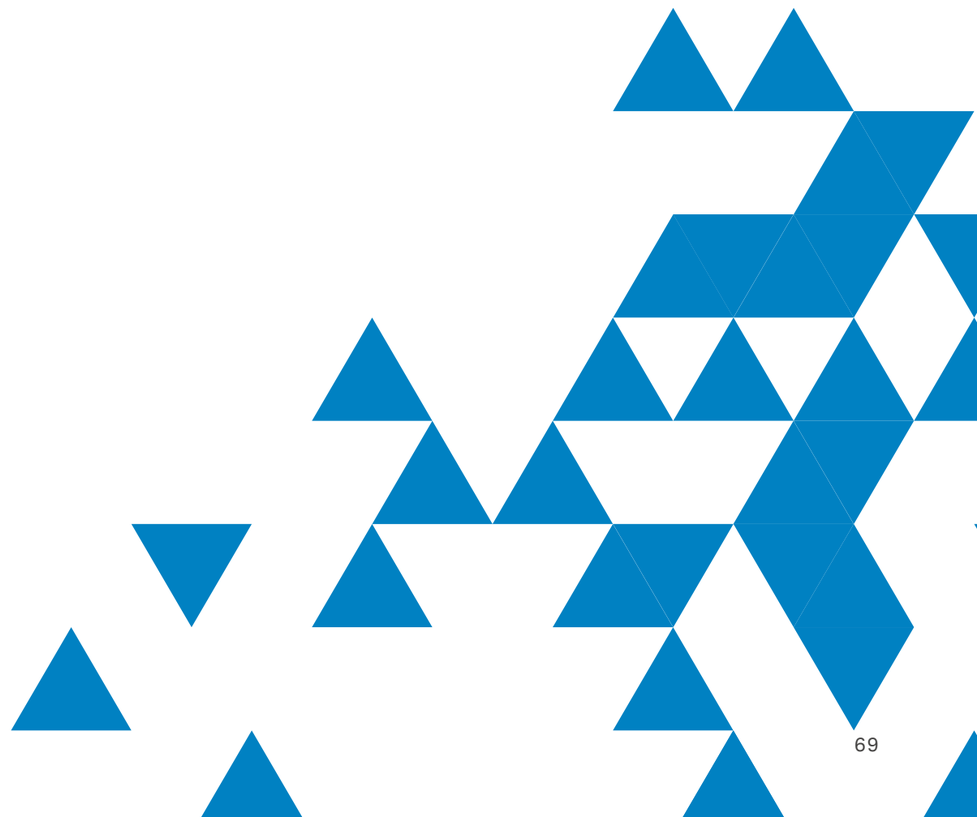
▪ Requirements for meningococcal polysaccharide vaccine	TRS 594	Annex 2	1975
▪ Addendum to TRS 594 Annex 2	TRS 658	Annex 6	1980
▪ Amendment to TRS 594 Annex 2	TRS 904	Annex 2	2002

86. This approach, rather than just producing an updated version of the original document, made the review process more challenging. This contrasts with the approach used by many regulators and pharmacopeia which publish their documents both as a web-based resource and a print edition. As updates to the monographs or guidelines are made, annotations to the revised versions to indicate where additions and deletions of text have taken place are published on their web sites. The EWG strongly recommends that WHO consider a similar approach in the maintenance of ECBS-adopted guidance documents to help ensure that the readers and users are aware of all current Committee recommendations. This approach would allow WHO to be more agile with revisions to their guidance documents and more easily adopt the latest advances in knowledge and practice.

[4] www.who.int/health-topics/biologicals

87. The deletion of the GST is another example of how the WHO's approach to managing changes to current practice can cause confusion. Although ECBS recommended the requirement for this test be removed from its guidelines in 2018 (WHO TRS. 2018;1016), 34 individual guidance documents from the 63 reviewed during the project still retain text indicating that the GST is recommended (p70). This raises the possibility that users of these guidance documents may still be performing the test. Responses to the manufacturers and regulators surveys we conducted in 2021 and 2022 respectively, indicated that 10% of manufacturers and 17% of NRAs who completed the survey were not aware that WHO had removed the requirement for this test. 50% of manufacturers were aware but nevertheless still performed the test and 67% of NRAs still received GST data in submissions. There may be many reasons for this but removal of all references to GST in current TRS documents would help to clarify the WHOs position and expectations for manufacturers and regulators.

88. Based on the observations from the working and focus groups of this project, it is strongly recommended that WHO and ECBS review its approach to publishing and curating their guidance documents published within the TRSs. It is suggested that a model which allows the online publication of the document that contains any amendments and revisions approved and published within the TRS should be adopted. A table at the beginning of each document could be used to indicate changes, dates when the changes were made, and citation to the publication in which ECBS approved the revision.



List of products where current TRS documents still includes a requirement for the GST

Dengue fever vaccines

Diphtheria vaccines

DT-Combined vaccines

Ebola vaccines

Haemophilus influenzae type B conjugate vaccines

Haemorrhagic fever vaccines

Hepatitis A vaccine (inactivated)

Hepatitis B vaccine (recombinant)

HPV

Human interferons

Influenza vaccines (inactivated)

Japanese encephalitis vaccine (inactivated)

Japanese encephalitis vaccines (live, attenuated)

Malaria vaccines

Meningococcal A conjugate vaccines

Meningococcal group C conjugate vaccines

MMR

Pertussis acellular vaccines

Pertussis vaccine whole cell

Plasmid DNA vaccines

Pneumococcal conjugate vaccines

Poliomyelitis vaccines (inactivated)

Rabies vaccine

Rift Valley Fever vaccine

Smallpox vaccine

Synthetic peptide vaccines

Tetanus vaccines

Tick-borne encephalitis vaccine (inactivated)

Typhoid conjugate vaccine

Typhoid vaccine (live, attenuated)

Typhoid vaccine polysaccharide

Varicella vaccine

Yellow fever vaccines

Snake antivenom immunoglobulins

Expectations for Stage Two of the project (WHO response to this report)

89. Due to the size and complexity of this project it was divided into two stages. This report represents the culmination of Stage One and will be presented to ECBS in October 2023. During Stage Two, WHO will formally respond to the report. We fully acknowledge that the WHO is not beholden to NC3Rs or the project working group to implement all or indeed any of the recommendations in this report. However, the participants of this project believe that the recommendations presented in this report are scientifically robust and would ultimately benefit those that manufacture, regulate and test vaccines and biological therapeutics. The shift towards non-animal technologies for quality control, batch release testing is widely accepted to be more scientifically relevant, more robust, faster and cheaper than traditional animal-based approaches. Indeed, there is a global trend towards adoption of the 3Rs and the working group believe that WHO should provide leadership and be proactive in driving the move away from animal-based testing where this is scientifically justifiable. The authors of this report are hopeful that WHO will implement most, if not all of their recommendations and would be happy to assist in Stage Two of the project if WHO would find this helpful.

Impact of recommendations on physical standards

90. The WHO has a number of international reference standards against which regional, national and international laboratories and manufacturers calibrate their own working standards. These standards are typically calibrated in units of biological activity, assigned following multi-laboratory collaborative studies. The standards are characterised and calibrated using one or more method types reflective of those included in regulatory guidelines and monographs – this includes animal methods for some standards. Where the working group makes recommendations for a shift from an animal-based assay to the use of non-animal technology, this may have implications for the approach to calibration of standards in the future. It may also create the need to develop new standards and reagents that will support development, implementation and use of non-animal methods.

91. Many of these international reference standards are produced and distributed by the Medicines and Healthcare products Regulatory Agency (MHRA) in the UK. The current list of WHO standards held by MHRA that currently require animals for characterisation and/or calibration is included in Annex 9. These standards may be impacted by the recommendations of this report and may need to be recalibrated against a non-animal-based assay going forward.

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55. Chabaud-Riou M *et al.* (2017). G-protein based ELISA as a potency test for rabies vaccines. *Biologicals*. 46: 124-9.

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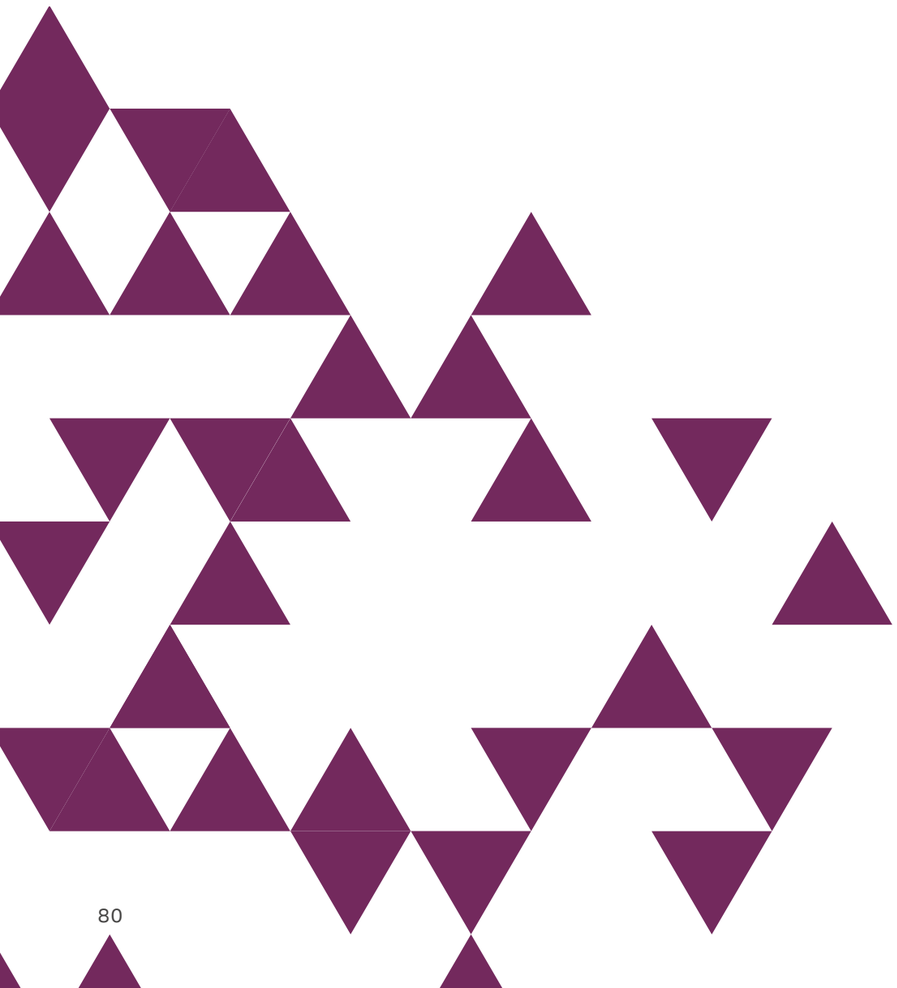
Annexes

Annex 1 – Table of animal tests and 3Rs language

All of the data, including the animal tests identified in the review with original and recommended new text, will be made available alongside this report via a **web-based resource** on the NC3Rs website.



Annex 2 – List of WHO TRS documents reviewed

81 documents have been reviewed, 63 mention animal testing or include 3Rs language relevant to the project

The documents highlighted in purple are those that do not mention animal testing or the 3Rs.

WHO guideline title	TRS/ Annex	Year	Animal testing/ 3Rs
General requirements for the sterility of biological substances	850 Annex 4	1973	N
Requirements for meningococcal polysaccharide vaccine	594 Annex 2	1975	Y
Requirements for meningococcal polysaccharide vaccine (addendum 1980)	658 Annex 6	1980	Y
Proposed requirements for Rift Valley Fever vaccine (inactivated) for Human Use	673 Annex 4	1981	Y
Requirements for typhoid vaccine (live attenuated, Ty 21a, oral)	700 Annex 3	1984	Y
Requirements for human interferons prepared from lymphoblastoid cells	786 Annex 3	1989	Y
Guidelines for national authorities on quality assurance for biological products	822 Annex 2	1992	N
Requirements for Vi polysaccharide typhoid vaccine	840 Annex 1	1994	Y
Requirements for varicella vaccine (live)	848 Annex 1	1994	Y
Haemorrhagic fever with renal syndrome (HFRS) vaccines (inactivated)	848 Annex 2	1994	Y
Requirements for measles, mumps, rubella vaccines and combined vaccine (live)	840 Annex 3	1994	Y
Regulation and licensing of biological products in countries with newly developing regulatory authorities	858 Annex 1	1995	N
Requirements for hepatitis A vaccine (inactivated)	858 Annex 2	1995	Y
Requirements for tick-borne encephalitis vaccine (inactivated)	889 Annex 2	1999	Y

WHO guideline title	TRS/ Annex	Year	Animal testing/ 3Rs
Guidelines for the production and quality control of synthetic peptide vaccines	889 Annex 1	1999	Y
Requirements for meningococcal polysaccharide vaccine (addendum 1999)	904 Annex 2	1999	Y
Recommendations for the production and control of haemophilus influenzae type b conjugate vaccines	897 Annex 1	2000	Y
Recommendations for the production and control of meningococcal group C conjugate vaccines	924 Annex 2	2001	Y
WHO Guidelines on Transmissible Spongiform Encephalopathies in relation to Biological and Pharmaceutical Products	n/a	2003	N
Recommendations for the production and control of meningococcal group C conjugate vaccines (addendum 2003)	926 Annex 3	2003	Y
Guidelines for the production and control of inactivated oral cholera vaccines	924 Annex 3	2004	Y
Guidelines on regulatory expectations related to the elimination, reduction or replacement of thiomersal in vaccines	926 Annex 4	2004	N
Recommendations for the production and quality control of smallpox vaccine (revised 2003)	926 Annex 1	2004	Y
WHO guidelines on nonclinical evaluation of vaccines	927 Annex 1	2005	N
Recommendations for the production and control of influenza vaccine (inactivated)	927 Annex 3	2005	Y
Recommendations for the preparation, characterization and establishment of international and other biological reference standards (revised 2004)	932 Annex 2	2006	N
Recommendations for whole-cell pertussis vaccine	941 Annex 6	2007	Y
Recommendations for inactivated rabies vaccine for human use produced in cell substrates and embryonated eggs	941 Annex 2	2007	Y
Guidelines to assure the quality, safety and efficacy of live attenuated rotavirus vaccines (oral)	941 Annex 3	2007	Y

WHO guideline title	TRS/ Annex	Year	Animal testing/ 3Rs
Recommendations for Japanese encephalitis vaccine (inactivated) for human use (revised 2007)	963 Annex 1	2011	Y
Recommendations to assure the quality, safety and efficacy of group A meningococcal conjugate vaccines	962 Annex 2	2011	Y
Part C. Clinical evaluation of group C meningococcal conjugate vaccines (revised 2007)	963 Annex 3	2011	
WHO Manual for the establishment of national and other secondary standards for vaccines	WHO/ IVB/11.03	2011	Y
Guidelines on stability evaluation of vaccines	962 Annex 3	2011	Y
Standard Operating Procedure: Mutant analysis by PCR and restriction enzyme cleavage (MAPREC) for oral poliovirus (Sabin) vaccine type 1, 2 or 3	n/a	2012	N
Standard Operating Procedure: Neurovirulence test of attenuated poliomyelitis vaccines (oral) in monkeys	n/a	2012	Y
Recommendations to assure the quality, safety and efficacy of BCG vaccines	979 Annex 3	2013	Y
Recommendations to assure the quality, safety and efficacy of pneumococcal conjugate vaccines	977 Annex 3	2013	Y
Recommendations to assure the quality, safety and efficacy of acellular pertussis vaccines	979 Annex 4	2013	Y

WHO guideline title	TRS/ Annex	Year	Animal testing/ 3Rs
Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks	978 Annex 3	2013	Y
Guidelines for independent lot release of vaccines by regulatory authorities	978 Annex 2	2013	Y
Manual for Quality Control of Diphtheria, Tetanus and Pertussis Vaccines	WHO/ IVB/11.11	2013	Y
Recommendations to assure the quality, safety and efficacy of influenza vaccines (human, live attenuated) for intranasal administration	977 Annex 4	2013	Y
Recommendations to assure the quality, safety and efficacy of recombinant hepatitis B vaccines	978 Annex 4	2013	Y
Recommendations to assure the quality, safety and efficacy of live attenuated yellow fever vaccines	978 Annex 5	2013	Y
Guidelines on the quality, safety and efficacy of dengue tetravalent vaccines (live, attenuated)	979 Annex 2	2013	Y
Generic protocol for the calibration of seasonal and pandemic influenza antigen working reagents by WHO essential regulatory laboratories	979 Annex 5	2013	Y
Methodological considerations: Potency tests for recombinant adjuvanted RTS,S vaccine	n/a	2013	Y
Guidelines on evaluation of similar biotherapeutic products (SBPs)	977 Annex 2	2013	N
Recommendations to assure the quality, safety and efficacy of Japanese encephalitis vaccines (live, attenuated) for human use	980 Annex 7	2014	Y
Guidelines on the nonclinical evaluation of vaccine adjuvants and adjuvanted vaccines	987 Annex 2	2014	Y
Recommendations to assure the quality, safety and efficacy of diphtheria vaccines (adsorbed)	980 Annex 4	2014	Y
Recommendations to assure the quality, safety and efficacy of tetanus vaccines (adsorbed)	980 Annex 5	2014	Y
Recommendations to assure the quality, safety and efficacy of poliomyelitis vaccines (oral, live, attenuated)	980 Annex 2	2014	Y

WHO guideline title	TRS/ Annex	Year	Animal testing/ 3Rs
Guidelines on the quality, safety and efficacy of recombinant malaria vaccines targeting the pre-erythrocytic and blood stages of Plasmodium falciparum	980 Annex 3	2014	Y
Guidelines on the quality, safety and efficacy of biotherapeutic protein products prepared by recombinant DNA technology	987 Annex 4	2014	N
Recommendations to assure the quality, safety and efficacy of DT-based combined vaccines	980 Annex 6	2014	Y
Scientific principles for regulatory risk evaluation on finding an adventitious agent in a marketed vaccine	993 Annex 2	2015	Y
Guidelines on procedures and data requirements for changes to approved vaccines	993 Annex 4	2015	N
Standard Operating Procedure: Neurovirulence test of types 1, 2 or 3 live attenuated poliomyelitis vaccines (oral) in transgenic mice susceptible to poliovirus	n/a	2015	Y
Recommendations to assure the quality, safety and efficacy of poliomyelitis vaccines (inactivated)	993 Annex 3	2015	Y
WHO good manufacturing practices for biological products	999 Annex 2	2016	Y
Guidelines on the stability evaluation of vaccines for use under extended controlled temperature conditions	999 Annex 5	2016	Y
Recommendations to assure the quality, safety and efficacy of recombinant human papillomavirus virus-like particle vaccines	999 Annex 4	2016	Y
Human challenge trials for vaccine development: regulatory considerations	1004 Annex 10	2017	N
Guidelines on clinical evaluation of vaccines: regulatory expectations	1004 Annex 9	2017	N
Guidelines on regulatory preparedness for provision of marketing authorization of human pandemic influenza vaccines in non-vaccine-producing countries	1004 Annex 7	2017	N

WHO guideline title	TRS/ Annex	Year	Animal testing/ 3Rs
Guidelines on evaluation of monoclonal antibodies as similar biotherapeutic products (SBPs)	1004 Annex 2	2017	Y
Guidelines for the production, control and regulation of snake antivenom immunoglobulins	1004 Annex 5	2017	Y
Recommendations for the production and control of influenza vaccine (inactivated) (addendum 2017)	1007 Annex 8	2017	Y
Guidelines on the quality, safety and efficacy of Ebola vaccines	1011 Annex 2	2018	Y
Guidelines on procedures and data requirements for changes to approved biotherapeutic products	1011 Annex 3	2018	N
WHO Questions and Answers: Similar Biotherapeutic Products. Complementary document to the WHO Guidelines on evaluation of similar biotherapeutic products (SBPs)	n/a	2018	N
Guidelines for the safe production and quality control of poliomyelitis vaccines	1016 Annex 4	2019	Y
Recommendations to assure the quality, safety and efficacy of recombinant hepatitis E vaccines	1016 Annex 2	2019	Y
Guidelines for the safe development and production of vaccines to human pandemic influenza viruses and influenza viruses with pandemic potential	1013 Annex 3	2019	N
Guidelines on the quality, safety and efficacy of respiratory syncytial virus vaccines	1024 Annex 2	2020	Y
Recommendations to assure the quality, safety and efficacy of poliomyelitis vaccines (inactivated) (addendum 2020)	1024 Annex 3	2020	Y
Recommendations to assure the quality, safety and efficacy of typhoid conjugate vaccines	1030 Annex 2	2021	Y
Recommendations to assure the quality, safety and efficacy of enterovirus 71 vaccines (inactivated)	1030 Annex 3	2021	Y
Guidelines on the quality, safety and efficacy of plasmid DNA vaccines	1028 Annex 2	2021	Y

Annex 3 – Publications and project dissemination

Publications arising from the project

Posters

- Poster presented at the 11th World Congress on Alternatives and Animal Use in the Life Sciences: Review of animal testing requirements in WHO guidelines and recommendations for biologics: a proposal to implement 3Rs principles.
- Poster presented at the 3rd International Alliance for Biological Standardization (IABS) Workshop focused on Global Harmonization of Specifications: Review of animal testing requirements in WHO guidelines and recommendations for biologics: a proposal to implement 3Rs principles.
- Poster presented at the 12th World Congress on Alternatives and Animal Use in the Life Sciences: Bringing quality control and batch release testing of biologicals into the 21st century.

Papers

- Lilley E *et al.* (2021) Integrating 3Rs approaches in WHO guidelines for the batch release testing of biologicals. *Biologicals* 74: 24-27. doi.org/10.1016/j.biologicals.2021.10.002
- Lilley E *et al.* (2023) Integrating 3Rs approaches in WHO guidelines for the batch release testing of biologicals: Responses from a survey of vaccine and biological therapeutics manufacturers. *Biologicals* 81: 101660. doi.org/10.1016/j.biologicals.2022.11.002
- Lilley E *et al.* (2023) Integrating 3Rs approaches in WHO guidelines for the batch release testing of biologicals: responses from a survey of National Control Laboratories and National Regulatory Authorities. *Biologicals*: *in press*

Meetings/conferences where the project has been presented

Date	Meeting name	Location	Presentation type
3 – 4 December 2019	IABS Meeting: Animal Testing for Vaccines – Implementing Replacement, Reduction and Refinement: Challenges and Priorities	Bangkok, Thailand	Oral
24 September 2020	DCVMN 3Rs group	Virtual	Oral

Date	Meeting name	Location	Presentation type
23 – 27 August 2020	11th World Congress on Alternatives and Animal Use in the Life Sciences	Virtual	Poster
15 September 2020	Wellcome Trust	Virtual	Oral
8 April 2021	African Academy of Sciences	Virtual	Oral
11 August 2021	AVAREF	Virtual	Oral
8 September 2021	DCVMN 3Rs group	Virtual	Oral
16 September 2021	EDQM group heads	Virtual	Oral
22 November 2021	WHO NCL-Network	Virtual	Oral
25 November 2021	VAC2VAC	Virtual	Oral
2 March 2022	Europe region stakeholder workshop	Virtual	Oral
4 April 2022	ECBS meeting	Virtual	Oral
28 April 2022	Asia region stakeholder workshop	Virtual	Oral
24 August 2022	RSPCA 4th International Focus on Severe Suffering meeting	Karolinska Institute, Sweden	Oral
26 September 2022	Pan-America region stakeholder workshop	Virtual	Oral
10 January 2023	IABS meeting: 3rd Workshop focused on Global Harmonization of Specifications	Basel, Switzerland	Poster
27 February 2023	The future of pyrogenicity testing: new approaches discussed at joint EDQM-EPAA event	Brussels, Belgium	Oral
5 July 2023	19th World Congress of Basic & Clinical Pharmacology 2023	Glasgow, UK	Oral
29 August 2023	12th World Congress on Alternatives and Animal Use in the Life Sciences	Niagara Falls, Canada	Oral

Annex 4 – Working group membership

Member	Affiliation	Review group	Survey development group	Start and end
Uzma Alam	Science for Africa Foundation	–	–	April 2021 – N/A
Cynthia Allen	Health Canada	–	–	June 2021 – N/A
Dave Allen	NICEATM	General	–	June 2020 – N/A
Patricia Aprea	ANMAT	General	–	June 2020 – September 2022
Cristina Barbirato	Merck	General	Manufacturers survey	June 2020 – N/A
Arun Bharadwaj	Central Drugs Laboratory	–	–	March 2022 – N/A
Martijn Bruysters	RIVM	Viral	NRA/NCL survey	June 2020 – N/A
Gilles Chénard	Johnson and Johnson	Viral	–	June 2020 – N/A
Emmanuelle Coppens	Sanofi Pasteur	General/Viral/ DTP	Manufacturers survey/NRA/ NCL survey	June 2020 – N/A
Wlamir Correa de Moura	FIOCRUZ / INCQS -BraCVAM	Viral	–	January 2021 – N/A
Angéle Costanzo	EDQM	Viral	–	June 2020 – September 2021
Pradip Das	Biological E	–	–	June 2021 – N/A
Blaise Descampe	GSK	DTP	–	January 2021 – September 2021
Francis Galaway	MHRA	–	–	December 2021 – N/A

Member	Affiliation	Review group	Survey development group	Start and end
Simeon Gill	Astra Zeneca	Bacterial	Manufacturers survey	June 2020 – N/A
Sunil Goel	Serum Institute	Viral/Bacterial	Manufacturers survey	June 2020 – N/A
Marlies Halder	EURL ECVAM	Bacterial	Manufacturers survey	June 2020 – April 2021
Anthony Holmes	NC3Rs (Project Lead)	–	–	June 2020 – N/A
Richard Isbrucker	WHO/Health Canada	General/ Bacterial/DTP	NRA/NCL survey	June 2020 – N/A
Masaaki Iwaki	Japanese National Institute of Infectious Diseases	Bacterial	NRA/NCL survey	June 2020 – N/A
David Jones	MHRA (Retired)	Viral	–	June 2020 – N/A
Carmen Jungbäck	IABS	General/Viral	–	June 2020 – N/A
Denis Lambrigts	GSK	DTP	Manufacturers survey	June 2020 – January 2021
Mario Landys Chovel Cuervo	Finlay Institute	Bacterial/DTP	Manufacturers survey	June 2020 – N/A
Elliot Lilley	NC3Rs (Programme Manager)	–	–	October 2020 – N/A
Derek Litthauer	South African Health Products Regulatory Authority	Viral	–	June 2020 – June 2021
Robin Levis	FDA	Viral	–	June 2020 – N/A
Laurent Mallet	EDQM	–	–	September 2021 – N/A
Sylvie Morgeaux	ANSM	Viral	–	January 2021 – N/A
Zebun Nahar	Incepta Vaccine	Viral	Manufacturers survey	October 2020 – N/A

Member	Affiliation	Review group	Survey development group	Start and end
Volker Öppling	Paul Erlich Institute	DTP	-	June 2020 – N/A
Supaporn Phumiamorn	Institute of Biological Products, Ministry of Public Health	Viral	NRA/NCL survey	June 2020 – N/A
Jean-Marie Préaud	IABS	–	–	February 2021 – N/A
Gayle Pulle	Health Canada	Viral	–	June 2020 – June 2021
Ian Ragan	NC3Rs (Chair)	General	–	June 2020 – N/A
Ute Rosskopf	WHO	Bacterial	–	June 2020 – February 2021
Mitsutoshi Senoh	Japanese National Institute of Infectious Diseases	DTP	NRA/NCL survey	June 2020 – N/A
Shahjahan Shaid	GSK	–	–	September 2021 – N/A
Sarah Sheridan	Merck	–	–	December 2021 – N/A
Dean Smith	Health Canada	Viral/DTP	–	June 2020 – N/A
Yeowon Sohn	Seoul National University	General	–	June 2020 – N/A
Paul Stickings	NIBSC	DTP	NRA/NCL survey	June 2020 – N/A
Joris Vandeputte	IABS	General	–	June 2020 – February 2021
Youchun Wang	National Institutes for Food and Drug Controls	Viral	NRA/NCL survey	June 2020 – N/A

Annex 5 – Focus group membership

Focus group	Member	Affiliation	WG or guest
Adventitious agents	Cristina Barbirato	Merck	WG
	Gwenael Cirefice	EDQM	Guest
	Gilles Chénard	Johnson and Johnson	WG
	Pradip Das	Biological E	WG
	Noémie Deneyer	GSK	Guest
	Carmen Jungbäck	IABS	WG
	Robin Levis	FDA	WG
	Carine Logvinoff	Sanofi	Guest
	Sylvie Morgeaux	ANSM	WG
	Shahjahan Shaid	GSK	WG
Neurovirulence	Martijn Bruysters	RIVM	WG
	Emmanuelle Coppens	Sanofi Pasteur	WG
	Robin Levis	FDA	WG
	Virginie Pithon	ANSM	Guest
	Tong Wu	Health Canada	Guest
Endotoxin/pyrogen	Dave Allen	NICEATM	WG
	Thierry Bonnevay	Sanofi	Guest
	Emmanuelle Charton	EDQM	Guest
	Eliana Coccia	ISS	Guest
	Marilena Etna	ISS	Guest

Focus group	Member	Affiliation	WG or guest
Endotoxin/pyrogen	Richard Isbrucker	WHO	WG
	Volker Öppling	Paul Erlich Institute	WG
	Octavio Presgrave	NCQS/Fiocruz	Guest
	Shahjahan Shaid	GSK	WG
	Paul Stickings	MHRA	WG
	Caroline Vipond	MHRA	Guest
Potency	Cynthia Allen	Health Canada	WG
	Ali Azizi	Health Canada	Guest
	Emmanuelle Coppens	Sanofi Pasteur	WG
	Wlamir Correa de Moura	FIOCRUZ / INCQS -BraCVAM	WG
	Angéle Costanzo	EDQM	Guest
	Pradip Das	Biological E	WG
	Richard Isbrucker	WHO	WG
	Sylvie Morgeaux	ANSM	WG
	Shahjahan Shaid	GSK	WG
	Paul Stickings	MHRA	WG
Specific toxicity	Dave Allen	NICEATM	WG
	Emmanuelle Coppens	Sanofi Pasteur	WG
	Angéle Costanzo	EDQM	WG
	Pradip Das	Biological E	WG
	Richard Isbrucker	WHO	WG
	Paul Stickings	MHRA	WG

Annex 6 – Endotoxin and pyrogen testing guidance

Justification for guidance document for endotoxin and pyrogen testing

1. The focus group recommends that ECBS establishes a drafting group to produce dedicated guidance around endotoxin and pyrogen testing which can be referenced in all TRS documents where such testing is currently recommended. This would provide important advice on transitioning to non-animal test methods and enable current best practice with regard to endotoxin and pyrogen testing (including new testing methodologies, cell lines and reagents) to be updated more efficiently. This section of the report outlines the rationale for this proposal and the key points that need to be included in an endotoxin and pyrogen testing guidance document.

Introduction

2. A brief introduction should include clear definitions of endotoxin and non-endotoxin pyrogens and an overview of the history of endotoxin and pyrogen testing. Sources of endotoxin and non-endotoxin pyrogens should be included. The introduction should also include a short description of the current animal-based testing methods (the rabbit pyrogen test; RPT and the limulus amebocyte lysate test; LAL), including the issues associated with each test and the rationale for transition towards alternative methods. This should include the fact that the RPT is a qualitative test and therefore there are no international reference standards for the assay. The ethical and animal welfare issues related to the use of horseshoe crab blood for the LAL should also be included – specifically the mortality rates following blood harvesting and the ecological impact of declining populations [66] since two species (Limulus polyphemus and Tachypleus tridentatus) are currently listed respectively as vulnerable and endangered (www.iucnredlist.org/search?query=horseshoe%20crab&searchType=species).

Alternative methods

3. This section should provide information about the current alternative methods. At the point of writing this report these include the monocyte activation test (MAT; as a universal pyrogen test) and the recombinant factor C assay (rFC; as a replacement for LAL as an endotoxin-specific test). For each test the assay should be briefly described with specific details about the scientific, economic and animal welfare advantages over the current animal-based test method and any challenges associated with setting up, using and interpreting the data with each assay.

4. For MAT, advantages include that the assay is fully quantitative and that use of human monocytes means that all relevant human toll-like receptors (TLR) are present making it a more robust assay and more representative of responses seen in humans. Challenges include assay duration, need for specialised equipment, cost of commercial kits, donor variability (if whole blood is used), access to human blood and current status, availability and cost of suitable cell lines.

5. For RFc, advantages include being fully sustainable since a recombinant protein is used and that the method is considered equivalent or superior to compendial bacterial endotoxins test methods [38, 67, 68]. Challenges include concerns around comparability and costs of different commercial kits.

WHO recommendation for pyrogenicity testing and endotoxin testing

6. The focus group also suggests that WHO includes language in the guidance to encourage the use of non-animal technologies (e.g. MAT and RFc) for pyrogen and endotoxin testing and to establish a timeline for the eventual phasing out of the RPT, following the example set by the European pharmacopeia and Brazilian National Council for the Control of Animal Experimentation (CONCEA).

Annex 7 – 3Rs guidance

Justification for 3Rs guidance to be produced by WHO ECBS

1. The working group proposed that, as well as reviewing and where necessary updating WHO guidance documents related to quality control, batch release testing of vaccines and biological therapeutics, WHO should also develop and publish a 3Rs guidance document. The objective of this document would be to clearly set out WHO's commitment to applying the most scientifically robust testing methods which reduce reliance on animals now and in the future. This section of the report outlines the rationale for this proposal and the key points that need to be included in a 3Rs guidance document.

2. The WHO is in a strong position to be a global thought leader and promote the scientific and economic benefits of the 3Rs principles to the scientific community globally. However, the majority of TRS documents currently lack statements which support the 3Rs, and where this is included, the language is inconsistent and focuses mostly on reducing animal use and transitioning towards adoption of non-animal methods. With the exception of TRS 1004 Annex 5 (Guidelines for the production, control and regulation of snake antivenom immunoglobulins), which includes some excellent statements about animal welfare, refinement opportunities that reduce animal suffering and the variability in *in vivo* data are almost entirely lacking.

3. The view of the working group has been presented to key stakeholders in two surveys (see Annex 3) and a number of regional stakeholder workshops (see Annex 8). The proposal that WHO develops and publishes specific guidance to support the adoption of 3Rs approaches in quality control and batch release testing received widespread support from all stakeholders.

4. The working group recommends that ECBS establish a drafting group to develop a guidance or policy document or manual setting out their expectations for manufacturers and regulators with regard to implementation of the 3Rs. We recommend that this document should contain the following:

   - **Introduction, including the history of the 3Rs principles**

     A brief introduction describing the history of the 3Rs principles, defining each 'R' and setting out their scientific, economical and animal welfare benefits. Whilst the overall drive is towards replacement of *in vivo* assays with non-animal approaches, the importance of optimising experimental design and maximising animal welfare should not be ignored. More detailed information about each of the 3Rs can be found in the **3Rs and their scientific benefits section**.

   - **WHO statement endorsing the 3Rs**

     It is important that the WHO uses its strong leadership position to encourage consideration of the 3Rs at all relevant stages in the development and production of vaccines and biological therapeutics, from non-clinical research and development through to routine quality control, batch release testing. There is a growing international awareness of the scientific, economic and animal welfare benefits of the 3Rs and, if these benefits are to be fully realised, the 3Rs need to be embedded in the guidance that defines international best practice. The working group recommends that WHO makes a clear statement acknowledging the scientific limitations of many of the current *in vivo* assays and that where *in vitro* approaches are available, they are potentially more scientifically relevant, more reproducible, more reliable and are likely to speed up access to important biological products for the patients that need them most. Where animal assays are still needed, it is important that experimental design is optimised to ensure that studies do not need to be repeated unnecessarily and that high levels of animal welfare are maintained to reduce animal suffering and to improve data quality.

   - **Examples of how the 3Rs can be applied in WHO TRS documents**

     The guidance should provide examples of how the 3Rs can be applied in WHO TRS documents in assays that require living animals. Several examples of how this can be achieved are presented throughout this report. There are multiple examples of replacement (e.g. use of the Monocyte activation test (MAT) and Bacterial Endotoxin test (BET) to replace the rabbit pyrogen test (RPT) in pyrogenicity testing) and reduction (e.g. use of single dilution assays for Diphtheria and Tetanus potency testing), but relatively few examples of refinement. The working group recommend that WHO sets out some general key principles around animal husbandry, housing and care that can contribute to higher standards of animal welfare. Several high quality and scientifically evidenced guidance documents exist which describe current best practice in animal welfare and these should be listed in any WHO guidance supporting the implementation of the 3Rs.

▪ Expectations for replacement of animal-based assays with non-animal alternatives

The WHO should set out general principles to advise NRAs on how to evaluate proposals from manufacturers to replace an animal-based test method with a non-animal approach as part of a product submission or post approval change. The general principles should also describe how manufacturers should approach the development and validation of *in vitro* methods when a substitution is envisaged. This guidance should broadly set out the key information required to allow for substitution of an established *in vivo* approach including the scientific rationale and mechanistic basis for the proposed alternative test method and evidence that it is fit for purpose. Direct, one-to-one assay comparison between the original *in vivo* assay and a proposed *in vitro* replacement may not be scientifically relevant because of the lack of historical validation of the *in vivo* test method and the likely difference in the way critical quality attributes are assessed between the two methodologies. It is also important to emphasise that a single *in vivo* assay may be substituted by multiple *in vitro* assays that fully characterise the critical quality attributes measured by the existing test.

The reliance approach [69, 70] – "whereby the National Regulatory Authority (NRA) in one jurisdiction may take into account and give significant weight to assessments performed by another NRA or trusted institution, or to any other authoritative information in reaching its own decision" [71] – is a powerful way to facilitate and accelerate acceptance of 3Rs approaches. This approach, as well as early dialogue between manufacturers and NRAs should be encouraged by WHO and included in the guidance.

▪ Deletion of outdated test methods

A currently required test method may be shown, through rigorous scientific evaluation, to have little or no scientific value. The ATT/GST/innocuity test is an example of this and has been deleted from several national pharmacopeia and by the WHO. It is conceivable that additional tests may be identified in the future that can be similarly deleted rather than substituted. The working group recommends that WHO sets out broad principles for the deletion of an assay (where scientifically justified).

▪ 3Rs resources

The working group recommends the following resources for up-to-date information on implementation of the 3Rs.

3Rs centres/consensus platforms[5]

Name	Country	Website address
ANZCCART – The Australian and New Zealand Council for the Care of Animals in Research and Teaching	Australia	anzccart.adelaide.edu.au
IC-3Rs – Innovation Centre–3Rs	Belgium	www.ic-3rs.org
BraCVAM – Brazilian Centre for Validation of Alternative Methods	Brazil	www.incqs.fiocruz.br
CCAC – Canadian Council on Animal Care	Canada	ccac.ca
CCAAM/CaCVAM – Canadian Center for Alternatives to Animal Methods	Canada	www.uwindsor.ca/ccaam
3Rs Centre Czech Republic	Czech Republic	www.szu.cz
The Danish 3R-Center	Denmark	en.3rcenter.dk
FC3R – French Center for the 3Rs	France	www.fc3r.com
BB3R – Berlin-Brandenburg research platform	Germany	www.bb3r.de
CAAT Europe	Germany	www.biologie.uni-konstanz.de/leist/caat-europe
Charité 3R	Germany	charite3r.charite.de/en
Einstein Centre 3R	Germany	www.ec3r.org/en
ICAR3R–3R Centre JLU Giessen	Germany	www.uni-giessen.de/fbz/zentren/icar3r
SAAR – Society for Alternatives to Animal Experiments	India	www.saae-i.org
Leibniz Alternatives at IUF Research Institute for Environmental Medicine	Germany	en.leibniz-alternatives.de
TARCforce3R	Germany	www.unimedizin-mainz.de/tarc-force-3r
3R Centre Rhine Neckar	Germany	en.3r-rn.de
JSAAE – The Japanese Society for Alternatives to Animal Experiments	Japan	www.asas.or.jp/jsaae/eng

[5] Adapted from eusaat.eu/the-3rs-society/3rs-associations-centers/3rs-europe

Name	Country	Website address
Luxembourg 3Rs Platform	Luxembourg	wwwen.uni.lu
3Rs – Centre of the Utrecht University and University Medical Centre Utrecht	Netherlands	www.uu.nl/en/organisation/3rs-centre
Norecopa	Norway	norecopa.no
ROCAM – Romanian Center for Alternative Test Methods	Romania	rocam.usamvcluj.ro
SNP3Rs – Slovak National Platform for 3Rs	Slovak Republic	www.snp3rs.com
CMCiB-IGTP–Comparative Medicine and Bioimage Centre of Catalonia, Germans Trias i Pujol Research Institute	Spain	www.cmcib.cat
Swedish 3Rs Center	Sweden	jordbruksverket.se/languages/english/the-swedish-3rs-center
3RCC – Swiss 3R Competence Centre	Switzerland	swiss3rcc.org
NC3Rs – National Centre for the 3Rs	UK	www.nc3rs.org.uk
3RCC - The 3Rs Collaborative	USA	www.na3rsc.org
NICEATM – NTP Interagency Center for the Evaluation of Alternative Toxicological Methods	USA	ntp.niehs.nih.gov/whatwestudy/niceatm/index.html
CAAT – Center for Alternatives to Animal Testing	USA	caat.jhsph.edu
ASCCT – American Society for Cellular and Computational Toxicology	USA	www.ascctox.org

Annex 8 – Regional stakeholder workshops

Organising committees

Europe workshop

- Dr Martijn Bruysters, National Institute for Public Health and the Environment (RIVM), The Netherlands
- Dr Emmanuelle Coppens, Sanofi, France
- Mr Simeon Gill, Medicines and Healthcare products Regulatory Agency (MHRA), UK
- Dr Sylvie Morgeaux, The National Agency for the Safety of Medicines and Health Products (ANSM), France
- Dr Volker Öppling, Paul-Ehrlich-Institut, Germany
- Dr Paul Stickings, Medicines and Healthcare products Regulatory Agency (MHRA), UK

Asia Workshop

- Dr Pradip Das, Biological E, India
- Dr Sunil Goel, Serum Institute, India
- Dr Masaaki Iwaki, National Institute of Infectious Diseases, Japan
- Dr Muthusamy Kalaivani, Indian Pharmacopoeia Commission, India
- Dr Zebun Nahar, Incepta Vaccine, Bangladesh
- Dr Supaporn Phumiamorn, Institute of Biological Products, Ministry of Public Health, Thailand
- Dr Jack Xie, Janssen, China
- Dr Aya Zamoto-Niikura, National Institute of Infectious Diseases, Japan

Pan-America Workshop

- Dr Cynthia Allen, Health Canada, Canada
- Dr Dave Allen, Integrated Laboratory Systems, USA
- Dr Maria Baca-Estrada, Health Canada, Canada
- Dr Wlamir Correa, ANVISA, Brazil
- Dr Eduardo Estrada, Sanofi, Mexico
- Mr Murilo Freitas, PAHO, USA
- Dr Robin Levis, FDA, USA
- Dr Mariluz Pombo, PAHO, USA
- Dr Octavio Presgrave, INCQS, Brazil
- Dr Dean Smith, Health Canada, Canada

Europe workshop – Wednesday 2 March 2022

Agenda

Welcome and introduction

Dr Ian Ragan, Chair of the NC3Rs working group to review animal use requirements guidelines

Opening address

Professor Klaus Cichutek, Paul-Ehrlich-Institut, Germany and outgoing Chair of ECBS

Scene setting

Project overview: Reviewing animal use requirements in WHO guidelines and opportunities for 3Rs approaches

Dr Elliot Lilley, NC3Rs

EDQM Achievements and Perspectives on 3Rs: Opportunities for WHO guidelines

Dr Laurent Mallet, Head of Department of Biological Standardisation, OMCL network & HealthCare

How aligned are we across Europe?

National perspectives on non-animal approaches for quality control and batch release testing of biologicals and the influence of WHO guidelines

- Dr Pavlinka Stoyanova, Bulgarian Drug Agency, Bulgaria
- Dr Volker Öppling, Head of Section Microbiological Vaccines, Paul-Erlich-Institut, Germany
- Dr Geneviève Waeterloos, Sciensano, Belgium

An industry consensus: How does (dis-)harmonisation in acceptance of 3Rs/non-animal approaches for quality control and batch release testing of biologicals impact manufacturers?

Dr Philippe Juvin & Dr Emmanuelle Coppens, Sanofi and representing IFPMA

Agenda

Panel session

Introduction to the session and voting instructions

Panel discussion

Moderator: Dr Svein Rune Andersen, Norwegian Medicines Agency, Norway

Panellists:

- Dr Emmanuelle Charton, EDQM
- Dr Shahjahan Shaid, GSK
- Dr Mark van Ooij, Janssen
- Dr Anne Dybwad, Norwegian Medicines Agency, Norway
- Dr Geneviève Waeterloos, Sciensano, Belgium
- Dr Paul Stickings, NIBSC, UK

The panel discussion will include opportunities for delegates to ask questions of the panel and to respond directly to questions themselves using an interactive voting system.

Wrap up and meeting close

Asia workshop – Thursday 28 April 2022

Agenda

Welcome and introduction

Dr Ian Ragan, Chair of the NC3Rs working group to review animal use requirements in WHO biologics guidelines

Aims and objectives for the meeting

Professor Yeowon Sohn, Seoul National University, Republic of Korea and member of the WHO Expert Committee on Biological Standardization

Scene setting

Project overview: Reviewing animal use requirements in WHO guidelines and opportunities for 3Rs approaches

Dr Elliot Lilley, NC3Rs

EDQM Achievements and Perspectives on 3Rs: Opportunities for WHO guidelines

Dr Laurent Mallet, Head of Department of Biological Standardisation, OMCL network & HealthCare

How aligned are we across Europe?

National perspectives on non-animal approaches for quality control and batch release testing of biologicals and the influence of WHO guidelines

- National Institutes for Food and Drug Control, China (TBC)
- Dr Koji Ishii, National Institute of Infectious Diseases, Japan
- Dr Rajeev Singh Raghuvanshi, Indian Pharmacopeia Commission, India
- Dr Huong Vu, National Institute for Control of Vaccines and Biologicals, Viet Nam
- Dr Wipawee Wongchana, Institute of Biological Products, Thailand

Manufacturers perspectives on the adoption of non-animal approaches in quality control & batch release testing of biologicals

- Dr Pradip Das, Biological E, India
- Dr Jack Xie, Janssen, China

Agenda

Panel session

Introduction to the session and voting instructions

Panel discussion

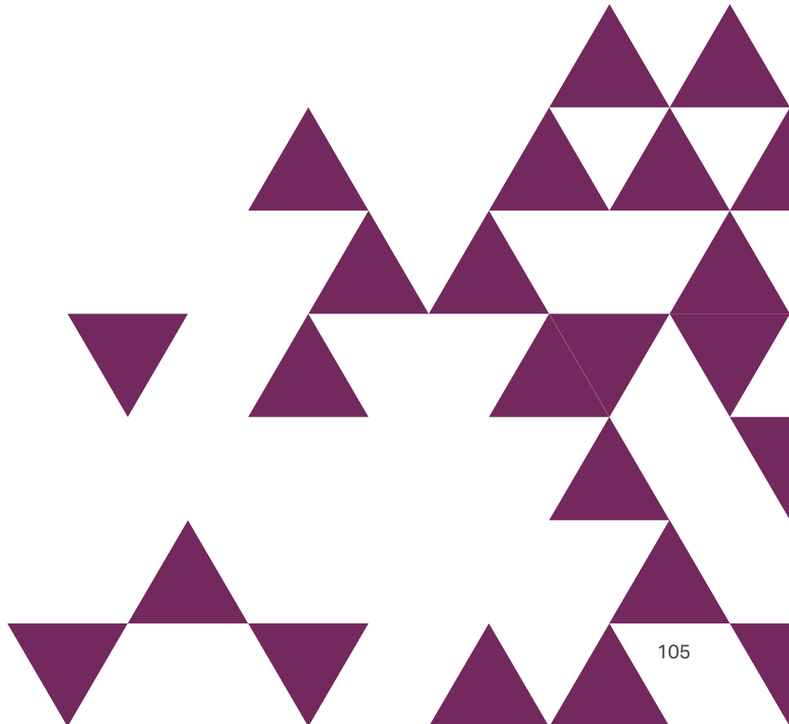
Moderator: (TBC)

Panellists:

- *Ms Ticha Kritsanaprasit, Queen Saovabha Memorial Institute, Thailand*
- *Dr Sunil Goel, Serum Institute India, India*
- *Dr Amrullah Aninditio, Biofarma, Indonesia*
- *Dr Elizabeth Ika Prawahju, Centre of National Quality Control Development of Drug and Food, Indonesia*
- *Dr Koji Ishii, National Institute of Infectious Diseases, Japan*
- *Dr Jongwon Kim, National Institute of Food and Drug Safety Evaluation, Republic of Korea*

The panel discussion will include opportunities for delegates to ask questions of the panel and to respond directly to questions themselves using an interactive voting system.

Wrap up and meeting close



Pan-America workshop – Monday 26 September 2022

Agenda

Welcome and introduction

Dr Ian Ragan, Chair of the NC3Rs working group to review animal use requirements in WHO biologics guidelines

Opening address

Dr Mario Landys, Instituto Finlay de Vacunas, Cuba

Scene setting

Project overview: Reviewing animal use requirements in WHO guidelines and opportunities for 3Rs approaches

Dr Elliot Lilley, NC3Rs

Opportunities and challenges for advancing 3Rs methods throughout Pan-America

Dr Maria Baca Estrada, Health Canada, Canada

Regulatory acceptance of non-animal approaches to replace *in vivo* assays for quality control and batch release testing of biologicals: science versus the fear factor

Dr Dean Smith, Associate Director, Centre for Biologics Evaluation, Health Canada, Canada

How does (dis-)harmonisation in acceptance of 3Rs/non-animal approaches for quality control and batch release testing of biologicals impact manufacturers in the region?

Dr Emmanuelle Coppens & Dr Philippe Juvin, Sanofi and on behalf of IFPMA

How aligned are we across Europe?

Panel 1 – Pyrogen/endotoxin testing

Scene setting introduction

Moderator: Dr Cynthia Allen, Health Canada, Canada

Panel discussion:

- *Dr Seeven Vydelingum, Sanofi Pasteur Vaccines, Canada*
- *Dr Gabriela Solano, Instituto Clodomiro Picardo, Costa Rica*
- *Dr Edith Lavado Perez, Centro Nacional de Control de Calidad, Instituto Nacional de Salud, Peru*
- *Dr Octavio Presgrave, INCQS, Brazil*
- *Dr Tong Wu, Health Canada, Canada*

Wrap up: Dr Cynthia Allen

Agenda

Panel 2 – Animal based potency and safety assays – a focus on DTaP/DTP products

Scene setting introduction

Moderator: Dr Rafael Baptista Silvestrin, Instituto Butantan, Brazil

Panel discussion (50 mins):

- *Dr Juthika Menon, Sanofi Pasteur Vaccines, Canada*
- *Dr Edson Sinuhe Torres Ballato, COFEPRIS, Mexico*
- *Dr Diana Mesa, National Institute for Drug and Food Surveillance (INVIMA), Colombia*
- *Dr Sayuri Reyes Garcia, Agencia Nacional de Regulacion, Control y Vigilancia Sanitaria, Ecuador*
- *Dr Gabriel Cabrejos, Centro Nacional de Control de Calidad, Instituto Nacional de Salud, Peru*
- *Dr Leslie Wagner, FDA, USA*
- *Dr Tong Wu, Health Canada, Canada*

Wrap up: Dr Rafael Baptista Silvestrin

Perspectives from a national pharmacopeia

Integrating 3Rs approaches in the Mexican Pharmacopeia and opportunities to support harmonization throughout Pan-America

MSc Angélica López Sotelo, Coordinator of Experts of the Biological Products Committee, Mexican Pharmacopeia

Wrap up and meeting close

Annex 9 – WHO International Standards held and distributed by MHRA

List of WHO international standards held and distributed by MHRA that require animals to source the material and/or for calibration/characterisation

No	Clinical Item Description	Allergen
84/685	Dog (Canis Familiaris) Hair Dander Extract. WHO International Standard	Allergen
PPDT	Purified Protein Derivative (PPD) of M. Tuberculosis Tuberculin WHO International Standard	Antigen
10/262	Diphtheria Antitoxin Human IgG (1st International Standard)	Antitoxin or Antiserum
13/240	2nd IS Tetanus Immunoglobulin Human	Antitoxin or Antiserum
18/180	2nd IS Diphtheria Antitoxin Equine	Antitoxin or Antiserum
2BADS	Anti-Brucella Abortus Serum, Bovine (International Standard)	Antitoxin or Antiserum
2CPBETAAT	Clostridium Perfringens Beta Antitoxin (2nd International Standard)	Antitoxin or Antiserum
2CPEPAT	Clostridium Perfringens Epsilon Antitoxin (2nd International Standard)	Antitoxin or Antiserum
61/001	Botulinum Antitoxin Equine Type D (1st International Standard)	Antitoxin or Antiserum
97/642	Bordetella pertussis anti serum (mouse) 1RR	Antitoxin or Antiserum
BUSB	Botulinum Antitoxin Equine Type B. WHO International Standard	Antitoxin or Antiserum
CDS	Anti-Canine Distemper Serum. WHO International Standard	Antitoxin or Antiserum
CHAN	Cholera Antitoxin, Goat. WHO International Standard	Antitoxin or Antiserum
CHS	Anti-Canine Hepatitis Serum	Antitoxin or Antiserum

No	Clinical Item Description	Allergen
DY	Anti-Dysentery (shiga) serum, Equine. WHO International Standard	Antitoxin or Antiserum
MGDS	Anti-Mycoplasma gallisepticum Serum. WHO Reference Reagent	Antitoxin or Antiserum
NDS	Anti-Newcastle Disease Serum. WHO International Standard	Antitoxin or Antiserum
OE	Gas Gangrene Antitoxin (Cl. novyi), Equine WHO International Standard	Antitoxin or Antiserum
PE	Gas Gangrene Antitoxin (Cl. Perfringens alpha antitoxin) Equine WHO International Standard	Antitoxin or Antiserum
QF	Anti-Q Fever Serum, Bovine. WHO International Standard	Antitoxin or Antiserum
SES	Swine Erysipelas Serum (anti-N) WHO Reference Reagent	Antitoxin or Antiserum
SFS	Anti-Swine Fever Serum. WHO International Standard	Antitoxin or Antiserum
SPDS-S2	Anti-Salmonella Pullorum Serum (Std. Form S) WHO International Standard	Antitoxin or Antiserum
SPDS-V	Anti-Salmonella Pullorum Serum (Variant Form V) WHO International Standard	Antitoxin or Antiserum
TE-3	Tetanus Immunoglobulin, Human WHO International Standard	Antitoxin or Antiserum
TILI	Anti-tick borne encephalitis serum (Louping Ill) WHO International Standard	Antitoxin or Antiserum
TISA	Anti-tick borne encephalitis serum (Sophyn & Absettarov) WHO International Standard	Antitoxin or Antiserum
VI	Gas Gangrene Antitoxin (Cl. Septicum), Equine WHO International Standard	Antitoxin or Antiserum
07/364	Chorionic Gonadotrophin, (5th IS)	Hormone
08/282	Follicle-Stimulating Hormone, human, recombinant, for bioassay (2nd International Standard)	Hormone
10/286	Follicle Stimulating Hormone, Luteinizing Hormone human, urinary for bioassay (5th International Standard)	Hormone
11/170	Erythropoietin, recombinant, for Bioassay (3rd International Standard)	Hormone
18/244	6th IS for hCG	Hormone
20/218	Follicle-Stimulating Hormone, human, recombinant, for bioassay (3rd IS)	Hormone
83/511	Insulin, Bovine. WHO International Standard	Hormone

No	Clinical Item Description	Allergen
83/515	Insulin, Porcine. WHO International Standard	Hormone
83/575	Follicle Stimulating Hormone, Pituitary. WHO International Standard	Hormone
84/514	Proinsulin, Bovine. International Ref. Preparation	Hormone
84/528	Proinsulin, Porcine. International Ref. Reagent	Hormone
86/690	Inhibin, Porcine. WHO International Standard	Hormone
92/510	Follicle Stimulating Hormone, Human, recombinant. WHO International Standard	Hormone
92/512	Follicle Stimulating Hormone, urofollitropin Human Urinary WHO International Standard	Hormone
96/602	Luteinizing Hormone, Human, recombinant. WHO International Standard	Hormone
15/106	Ancrod	Protease
RBT/16	Thromboplastin, Rabbit, Plain 5th International Standard 2016	Tissue Factor
15/126	Bordetella pertussis toxin (20µg). (2nd International Standard)	Toxin
STT	Diphtheria (Schick) Test Toxin WHO International Standard	Toxin
07/216	Diphtheria Toxoid (Adsorbed) (4th International Standard)	Vaccine
08/218	Tetanus Toxoid Adsorbed (4th International Standard)	Vaccine
12/104	International Standard for IPV 12/104 (3rd International Standard)	Vaccine
16/204	7th WHO International Standard for Rabies Vaccine	Vaccine
17/160	Sabin Inactivated Polio Vaccine (sIPV) (WHO 1st international Standard)	Vaccine
90/534	Mumps Vaccine (Live) WHO Reference Reagent	Vaccine
91/688	Rubella Vaccine (Live) 1st International Reference Reagent	Vaccine
92/648	Measles Vaccine (Live) 2nd International Reference Reagent	Vaccine
94/532	Pertussis Vaccine (Whole Cell) WHO International Standard	Vaccine
JNIH-3	Acellular Pertussis Vaccine - 1st IS.	Vaccine
SMV	Smallpox Vaccine WHO International Standard	Vaccine

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