Recommendations to assure the quality, safety and efficacy of poliomyelitis vaccine (oral, live, attenuated)


NOTE:

This document has been prepared for the purpose of inviting comments and suggestions on the proposals contained therein, which will then be considered by the Expert Committee on Biological Standardization (ECBS). Publication of this early draft is to provide information about the proposed document—Recommendations to assure the quality, safety and efficacy of poliomyelitis vaccine (oral, live, attenuated)—Proposed replacement of Annex 2 of WHO Technical Report Series, No. 980, to a broad audience and to ensure the transparency of the consultation process.

The text in its present form does not necessarily represent the agreed formulation of the ECBS. Written comments proposing modifications to this text MUST be received by 15 August 2022 using the Comment Form available separately and should be addressed to the Department of Health Products Policy and Standards, World Health Organization, 20 Avenue Appia, 1211 Geneva 27, Switzerland. Comments may also be submitted electronically to the Responsible Officer: Dr Tiequn Zhou at email: zhout@who.int.

The outcome of the deliberations of the Expert Committee will be published in the WHO Technical Report Series. The final agreed formulation of the document will be edited to be in conformity with the "WHO style guide, second edition" (KMS/WHP/13.1).
Recommendations to assure the quality, safety and efficacy of poliomyelitis vaccine (oral, live, attenuated)


Introduction

Purpose and scope

Terminology

General considerations

International reference materials

Part A.  Manufacturing recommendations

A.1  Definitions
A.2  General manufacturing recommendations
A.3  Control of source materials
A.4  Control of vaccine production
A.5  Filling and containers
A.6  Control tests on final lot
A.7  Records
A.8  Retained samples
A.9  Labelling
A.10  Distribution and transport
A.11  Stability testing, storage, and expiry date

Part B.  Nonclinical evaluation of poliomyelitis vaccines (oral, live, attenuated)

B.1  Characterization of a new Sabin virus sub-master seed
B.2  Characterization of virus seeds for the production of nOPV
B.3  Evaluation of immunogenicity of nOPV in suitable models

Part C.  Clinical evaluation of poliomyelitis vaccines (oral, live, attenuated)

C.1  General considerations
C.2  Safety and immunogenicity studies
C.3  Post-marketing studies and surveillance

Part D.  Recommendations for NRAs

D.1  General recommendations
D.2  Official release and certification

Part E.  Recommendations for poliomyelitis vaccines (oral, live, attenuated) prepared in primary monkey kidney cells

E.1  Control of vaccine production

Authors and acknowledgements

References
Appendix 1
Overview of virus seeds used in OPV production

Appendix 2
In vivo tests for neurovirulence, and considerations in relation to assay choice

Appendix 3
Preparation of poliomyelitis vaccines (oral, live, attenuated) using cell banks – example of a flowsheet

Appendix 4
Cell-culture techniques for determining the virus content of poliomyelitis vaccines (oral, live, attenuated)

Appendix 5
Model summary protocol for the manufacturing and control of poliomyelitis vaccines (oral, live, attenuated)

Appendix 6
Model certificate for the release of poliomyelitis vaccines (oral, live, attenuated) by NRAs

Appendix 7
Preparation of poliomyelitis vaccines (oral, live, attenuated) using primary monkey kidney cells – example of a flowsheet

Appendix 8
International reference materials for poliomyelitis vaccines (oral, live, attenuated)

Recommendations published by WHO are intended to be scientific and advisory in nature. Each of the following sections constitutes recommendations for national regulatory authorities (NRAs) and for manufacturers of biological products. If an NRA so desires, these WHO Recommendations may be adopted as definitive national requirements, or modifications may be justified and made by the NRA. It is recommended that modifications to these Recommendations be made only on condition that such modifications ensure that the vaccine is at least as safe and efficacious as that prepared in accordance with the Recommendations set out below. The parts of each section printed in small type are comments or examples intended to provide additional guidance to manufacturers and NRAs.
Introduction

Requirements for oral poliomyelitis vaccine (OPV) were first formulated in 1962 (1) and revised in 1966 (2) and 1972 (3) when an appendix describing the production of OPV in human diploid cells was added. The requirements were further updated in 1982 (4) following an accumulation of data, particularly on the performance and evaluation of the monkey neurovirulence test (MNVT) and tests on the karyology of human diploid cells. The Requirements for poliomyelitis vaccine (oral) were updated in full in 1989 (5) to take account of the general requirements for the characterization of continuous cell lines for the preparation of biologicals which were adopted in 1985 (6), and after a WHO Study Group concluded that, in principle, such cell lines are acceptable as substrates for the production of biologicals (7). An addendum was subsequently adopted (8) that introduced changes in tests for freedom from detectable DNA sequences of Simian virus 40 (SV40), introduced the mutant analysis by polymerase chain reaction (PCR) and restriction enzyme cleavage (MAPREC) assay as an optional additional in vitro test for poliovirus type 3, increased levels of laboratory containment for wild polioviruses (WPVs); and provided guidance on additional antibody screening tests (for foamy viruses) for animals from closed primate colonies used as a source for primary monkey kidney cells.

The Requirements (now Recommendations) were subsequently revised in full in 1999 (9) when the use of transgenic mice expressing the human poliovirus receptor (TgPVR21 mice) (10) as an alternative to the MNVT for type 3 virus was included in the revision and MAPREC test was introduced as the in vitro test of preference for the evaluation of filtered bulk suspensions for poliovirus type 3 (11). The previously mandated reproductive capacity at elevated temperature (rct40) test then became an optional, additional test if MAPREC test was performed. The studies with poliovirus types 1 and 2 in TgPVR21 mice were completed by June 2000, and an addendum to the Recommendations for the production and control of poliomyelitis vaccine (oral) was adopted in 2000 (12) that included the neurovirulence test in TgPVR21 mice (TgmNVT) as an alternative to the MNVT for all three poliovirus serotypes.

In 2012 the Recommendations were last revised in full to update on the origin of different virus strains for OPV production with inclusion of a new Appendix 1 and include consideration of new vaccine formulations (monovalent OPV- mOPV and bivalent OPV-bOPV) (13). The new Recommendations contained updated sections on international standards and reference preparations, general manufacturing recommendations and control tests, and the WHO standard operating procedures (SOPs) for TgmNVT and MAPREC in light of current developments in techniques. The document also included new sections on nonclinical and clinical evaluation of OPV, an update on terminology, and the introduction of the “virus sub-master seed lot” concept applicable only to the virus master seed supplied by WHO. Finally, it updated information on neurovirulence tests (MNVT and TgmNVT) and the MAPREC test which is extended to all three types of virus seeds and vaccine bulks; and inclusion of a new Appendix 2 giving the rationale for the choice of monkey or mouse neurovirulence tests.
Since then, there has been significant progress in global polio eradication as well as advances in scientific knowledge, novel laboratory techniques, including high-throughput sequencing (HTS, also known as next generation sequencing, massively parallel, or deep sequencing), and new non-pathogenic strains of polioviruses for quality control tests have become available. Wild-type polioviruses of serotypes 2 and 3 were declared eradicated globally by WHO in 2015 and 2019, respectively. Consequently, in April-May of 2016 the global use of trivalent OPV formulation (tOPV) for routine immunizations was replaced by the exclusive use of bOPV containing only serotypes 1 and 3. Therefore, manufacture of tOPV was discontinued, and now bOPV is being used for routine and supplemental immunization. Monovalent OPV of serotype 2 (mOPV2) is used to control outbreaks of type 2 circulating vaccine-derived poliovirus (cVDPV2). In addition, tOPV was approved by the Strategic Advisory Group of Experts on Immunization (SAGE) for use in control of type 2 cVDPV outbreaks occurring alongside WPV1 circulation. Rationally designed, more-genetically-stable strains of Sabin 2 virus were developed to minimise reversion of the vaccine strain to virulence and have been used to manufacture novel OPV2 (nOPV2). At the end of 2020 nOPV2 was introduced for cVDPV2 outbreak control under the WHO Emergency Use Listing (EUL) (14).

Other new WHO guidance documents were issued since the last revision of WHO Recommendations for OPV in 2012. A WHO Global Action Plan to minimize poliovirus facility-associated risk after type-specific eradication of wild polioviruses and sequential cessation of oral polio vaccine use (GAPIII) was adopted in 2014 (15). It tightened biosafety and biosecurity requirements for handling live polioviruses and led to the adoption of new Guidelines for the safe production and quality control of poliomyelitis vaccines in 2018 (16), and a subsequent amendment in 2020 (17). In 2020, the WHO Expert Committee on Biological Standardization (ECBS) concluded that the 2012 Recommendations for OPV should be revised. WHO convened a drafting group composed of regulators from several countries to prepare the draft of revision of TRS 980, Annex 2. A virtual informal consultation meeting was held by WHO on 15-17 November 2021. It was attended by experts and representatives from academia, national regulatory authorities (NRAs)/national control laboratories (NCLs), industry and other international health organizations and institutions involved in the research, manufacture, authorization, and testing/release of OPV from countries around the world to discuss and reach consensus on the issues in the revision (18).

Major issues addressed during this revision include:
− the use of HTS in quality control of OPV as an alternative to MAPREC test as a preferred in vitro test.
− analysis of whole genome mutational profiles generated by HTS as a possible future replacement of MNVT and TgmNVT for routine lot release after the manufacture consistency has been established. Practical experience in these areas is currently limited. Further guidance will be provided in due course.
− removal of the rct40 test because it is insufficiently sensitive and requires WPVs as control strains, which complicates GAPIII compliance.
− considerations of the design, manufacture, and quality control of nOPV strains.
− use of new non-pathogenic strains for measurement of neutralizing antibodies to polioviruses.
− updates on international reference materials relevant to OPV manufacture and control and inclusion of a new appendix on that.
− updates on Terminology.
− introduction of “virus sub-master seed” concept for nOPV strains in addition to Sabin OPV.
− guidance for clinical evaluation of new OPV vaccine strains that may be developed following OPV cessation.

Additional changes have been made to refer to and align the document with other WHO recommendations published since the last revision.

**Purpose and scope**

These WHO Recommendations provide guidance to NRAs and manufacturers on the manufacturing processes, quality control and nonclinical and clinical evaluations needed to assure the quality, safety and efficacy of live attenuated polio vaccines (oral).

The scope of these recommendations encompasses live attenuated polio vaccines (oral) derived from the original Sabin strains, some by simple passage and others by more complex routes, including plaque purification. This document is intended to apply to all OPV products prepared from Sabin poliovirus strains and their derivatives.

The recommendations also include consideration of issues raised in the manufacture and control of nOPV made from rationally designed strains created by targeted genetic manipulation of Sabin viruses and the introduction of HTS as a quality control method in a regulatory setting for both nOPV and Sabin OPV.

In the current document, OPV refers to oral polio vaccines made from any attenuated poliovirus, both the original Sabin strain and novel, genetically modified strains. In some cases, Sabin OPV and nOPV are used intentionally to distinguish between classical OPV and novel OPV.

These WHO Recommendations should be read in conjunction with other relevant WHO guidance documents such as that on nonclinical (19) and clinical (20) evaluation of vaccines, good manufacturing practices for biological products (21), characterization of cell banks (22), lot release (23), and guidelines for the safe production and quality control of poliomyelitis vaccines (16, 17).

**Terminology**

The definitions given below apply to the terms as used in these WHO Recommendations. These terms may have different meanings in other contexts.
Adventitious agents: contaminating microorganisms of the cell substrate or source materials used in their cultures, that may include bacteria, fungi, mycoplasmas, and endogenous and exogenous viruses that have been unintentionally introduced.

Cell-culture infective dose 50% (CCID$_{50}$): the quantity of a virus suspension that will infect 50% of cell cultures.

Cell seed: a quantity of vials containing well-characterized cells derived from a single tissue or cell of human or animal origin stored frozen in liquid nitrogen in aliquots of uniform composition, one or more of which would be used for the production of a master cell bank.

Comparator vaccine: an approved vaccine with established efficacy or with traceability to a vaccine with established efficacy that is tested in parallel with an experimental vaccine and serves as an active control in nonclinical or clinical testing.

Final bulk: the finished vaccine from which the final containers are filled. The final bulk may be prepared from one or more monovalent bulks and may contain more than one virus type.

Final lot: a collection of sealed final containers of finished vaccine that is homogeneous with respect to the risk of contamination during the filling process. All the final containers must therefore have been filled from a single vessel of final bulk in one working session.

Master cell bank (MCB): a quantity of fully characterized cells of human or animal origin frozen at $\leq$70 °C or below in aliquots of uniform composition, derived from the cell seed. The MCB is itself an aliquot of a single pool of cells, dispensed into multiple containers and stored under defined conditions. The MCB is used to derive all working cell banks. The testing performed on a replacement MCB – derived from the same cell clone, or from an existing master or working cell bank (WCB) – is the same as that for the initial master cell bank unless a justified exception is made.

Monovalent bulk: a pool of a number of single harvests of the same virus type processed at the same time.

High throughput sequencing (HTS): a next-generation sequencing technology based on sequencing of individual nucleic acid molecules that allows each nucleotide to be sequenced multiple times (massively parallel or deep sequencing), thereby enabling the detection and quantitation of sequence heterogeneities including single nucleotide polymorphisms (SNP).

Novel OPV (nOPV): rationally designed genetically modified derivatives of the live-attenuated Sabin vaccine strain. nOPV has enhanced genetic stability and lower risk of reversion to neurovirulence compared to the original Sabin strain.
Production cell culture: a cell culture derived from one or more ampoules of the WCB or primary tissue used for the production of vaccines.

RSO: RNA-plaque-derived Sabin Original type 3 virus (24). All subsequent passages are designated by an additional number – e.g., RSO1 (master seed) is one passage on from RSO. It is distributed to vaccine manufacturers that create their own master/sub-master and working seed stocks used for manufacture of monovalent bulks of OPV3.

Sabin strains: preparations of attenuated polioviruses of types 1, 2 and 3 derived by limited number of passages from stocks developed by Dr Albert Sabin (25) which retain attenuated properties as measured by biological and molecular markers.

Single harvest: a quantity of virus suspension of one virus type harvested from cell cultures derived from the same WCB and prepared from a single production run.

SO: Sabin Original virus as described in Sabin and Boulger 1983 (25). All subsequent passages are designated by an additional number – e.g. SO+1 is one passage on from Sabin Original.

Virus master seed lot: a quantity of virus suspension that has been processed at the same time to assure a uniform composition and has been characterized to the extent necessary to support development of the virus working seed lot. The characterized virus master seed lot is used for the preparation of virus working seed lots or a virus sub-master seed (if applicable).

Virus sub-master seed lot: a quantity of virus suspension produced by a single passage from the virus master seed and made at a multiplicity of infection that ensures the development of cytopathic effect within an appropriate timeframe, and that has been processed at the same time to assure a uniform composition. Sub-master seeds should be made by the manufacturer when the supply of well characterised master seed of Sabin OPV supplied by WHO is insufficient to meet production needs. They may also be produced from qualified nOPV master seeds if it is necessary. The virus sub-master seed lot should be characterized as extensively as the virus master seed lot to support the development of the virus working seed lot. The characterized virus sub-master seed lot is used for the preparation of virus working seed lots (see section A.3.2.2 and Part B).

Virus working seed lot: a quantity of virus of uniform composition, fully characterized, derived by only one passage from master or sub-master virus seed lot made at the multiplicity of infection, ensuring that cytopathic effect develops within an appropriate timeframe (e.g. three days) from a virus master seed lot or sub-master seed lot approved by the NRA for the manufacturing of vaccine.

Working cell bank (WCB): a quantity of cells of uniform composition derived from one or more ampoules of the MCB at a finite passage level, stored frozen at –70 °C or below in
aliquot, one or more of which would be used for vaccine production. All containers are treated identically and once removed from storage are not returned to the stock.

**General considerations**

Poliomyelitis is an acute communicable disease of humans caused by three distinct poliovirus serotypes called types 1, 2 and 3 distinguished by neutralization with type specific antibodies (26). Poliovirus is a species C human enterovirus of the Picornaviridae family and consists of a single-stranded, positive-sense RNA genome and a protein capsid.

Where sanitation is poor, these viruses are believed to spread mainly by faecal-to-oral transmission, whereas the oral-to-oral mode of transmission probably dominates in areas with a higher standard of sanitation. Mixed patterns of transmission are likely to occur in most settings. In the pre-vaccine era, roughly one out of 200 susceptible individuals infected by polioviruses developed paralytic poliomyelitis, while the rest were asymptomatic or had mild symptoms (26).

Progress in polio control (and, since 1988, polio eradication) has been mainly due to widespread use of vaccines. An inactivated poliovirus vaccine (IPV Salk vaccine) was licensed in 1955. The use of live, attenuated OPV (Sabin vaccine) for mass immunizations started in the Soviet Union and few other countries in 1959. It was licensed in the United States and some European countries as monovalent OPV in 1961, and as trivalent OPV (tOPV) in 1963. The Sabin strains of poliovirus used in the production of Sabin OPV were shown to be both immunogenic and highly attenuated when administered orally to susceptible children and adults. Most countries that initially introduced vaccination with IPV later changed to OPV because of ease of administration, suitability for mass vaccination campaigns, induction of superior intestinal mucosal immunity, and lower production costs. In 1974, OPV was recommended as part of the Expanded Programme on Immunization (EPI), and OPV was again the vaccine of choice in 1988 when the World Health Assembly resolved to eradicate polio globally by the year 2000. The last cases of poliomyelitis caused by WPV type 2 (WPV2) and 3 (WPV3) were reported in October 1999 in India and November 2012 in Nigeria respectively. Subsequently, the global eradication of WPV2 and WPV3 was certified on 20 September 2015 and 24 October 2019, respectively (26). By the end of 2021, WPV1 only remained endemic in two countries – Afghanistan and Pakistan.

Although OPV is a safe vaccine, adverse events may occur on rare occasions (26). Vaccine-associated paralytic poliomyelitis (VAPP) is the most important of these rare adverse events and is clinically indistinguishable from poliomyelitis caused by WPV. Identification of VAPP requires laboratory analysis of the virus isolated from the case. The incidence of VAPP has been estimated at 2–4 cases per million annual birth cohort in countries using OPV (26). Sabin viruses can spread in populations where the coverage of OPV is low. In such situations, Sabin viruses can acquire the neurovirulence and transmissibility characteristics of WPV, thus becoming cVDPV that can cause outbreaks of the disease (27), presenting a significant challenge to the global eradication campaign. cVDPV2 is the predominant type, and its continued circulation is
fuelled by inadequate population immunity. To prevent gaps in population immunity the switch from tOPV to bOPV (containing only vaccine viruses of serotypes 1 and 3) was supposed to be accompanied by the introduction of supplemental immunization with trivalent IPV. However, the shortage of IPV in some countries led to the decline in population immunity to type 2 poliovirus and the increase of cVDPV2 cases from 2 in 2016 to over 1,000 in 2020. Control of cVDPV2 outbreaks has been performed with targeted use of mOPV2, but where the campaigns have been poor because of difficulties in delivery they have triggered the emergence of new cVDPV2 outbreaks. Outbreaks due to type 1 and 3 cVDPVs have also occurred but to a lesser extent and continue to occur in recent years.

cVDPVs will continue to emerge as long as classical Sabin OPV is used and gaps in population immunity exist. To overcome this problem, in 2011 an international consortium of scientists sponsored by the Bill and Melinda Gates Foundation set out to develop novel vaccine strains having reduced risk of losing attenuated phenotype and evolving to neurovirulent cVDPVs. One of the resulting viruses has been used to produce an nOPV2 which has been granted EUL by WHO for use in type 2 cVDPV outbreaks (28-31). Additional strains could be developed in the future, including similarly genetically stabilized strains of serotypes 1 and 3. The design of the novel strains is based on understanding of the molecular biology of polio viruses and vaccines that has been gained over the years. The attenuation of the Sabin strains is associated in part with a highly base-paired hairpin structure in the 5'- non-coding region of the virus, called domain V, which is involved in the initiation of protein synthesis. The three Sabin strains have less thermally stable structures of this domain compared to respective wild strains, as a result of the introduction of a single base change in this section of the RNA which is different for each serotype, but which changes the strength of a base pair. As it is a single base change, all three serotypes can readily revert by a single mutational event to the wild type sequence at this position and this is observed in vaccine recipients. Viruses have therefore been constructed which make it harder for the hairpin structure of domain V become stronger by mutation. This was done by replacing stronger GC pairs and weaker GU pairs with intermediately strong AU pairs, so that the overall thermostability of the hairpin and therefore the virus neurovirulence remained unchanged. However, this made the attenuated phenotype more stable because in this re-designed structure two simultaneous mutations at any given position are required to revert to the wild type base pair strength. The nOPV strains should therefore be at least as attenuated as the Sabin strains and genetically more stable. This was demonstrated to be the case both in vitro, in animal models and in human trials. Modifications were also introduced into the viral polymerase to increase the virus genetic stability by reducing the mutation and recombination rates. In addition, an essential cis-acting replicative element has been moved from the centre of the genome to the 5'- UTR to minimise the risk of removing the genetically modified domain V region by recombination.

The key to nOPV safety lies in the low level of reversion at key known sites. Consistency has been monitored by molecular means rather than animal tests, although animal tests are retained as a final check. The nOPV strains have different properties to the classical Sabin strains with respect to optimal growth conditions, therefore production and quality control of vaccines made from the new strains may differ in detail from those used for the classical Sabin strains. This
includes growth and titration properties, optimal temperature of growth, dose required, thermal
stability and other parameters. The nucleotide sequence of nOPV2 strain is available in GenBank
(accession number MZ245455) and the graphic representation of its structure is shown in Figure
3 in Appendix 1. Similar nOPV1 and nOPV3 strains are currently in early clinical development
and in the future may be used in trivalent formulations. The novel strains are of great importance
to the eradication programme and are considered in this revision.

Trivalent formulations of conventional (Sabin) OPV were created in the early 1960s to ensure
that the immune response against all three poliovirus serotypes was adequate. Subsequent studies
demonstrated that the Sabin 2 virus had higher fitness and interfered with the immunogenicity of
serotypes 1 and 3, leading to lower seroconversion (32). In 2008, a clinical trial to evaluate the
immunogenicity of alternative OPV formulations (mOPV1, mOPV3 and bOPV) compared to
tOPV was conducted by WHO in the Indian population. The seroconversion rates to poliovirus
type 1 and type 3 following immunization with bOPV were significantly higher than that
induced by tOPV and were not lower than those induced by immunization with either mOPV1 or
mOPV3 alone (33). The introduction and widespread use of mOPV1 and mOPV3 in
supplementary immunization activities in 2005 resulted in substantial reductions of cases caused
by the respective serotypes. This led to the stopping of WPV1 circulation in India, and WPV3
eradication worldwide, which was declared in 2019. However, continued circulation of WPV1 in
two remaining polio-endemic countries requires huge quantities of bOPV to be given in routine
and mass campaigns conducted in 140 countries throughout the world.

In addition to bOPV, which is used in most countries for routine or supplementary vaccination,
monovalent OPVs of all three serotypes are used by the Global Polio Eradication Initiative
(GPEI)¹ and have been licensed for use in endemic countries or for outbreak control in situations
where one or two types can re-emerge. In 2020 SAGE recommended that tOPV be made
available to countries for cVDPV2 outbreak response in subnational areas where there is co-
circulation or high risk of co-circulation of cVDPV2 with cVDPV1, cVDPV3 or WPV1 instead
of dual mOPV2 and bOPV campaigns (34). Therefore, at the moment there is a need for all
known formulations of OPV.

Live vaccines prepared from Sabin poliovirus strains of types 1, 2, and 3 were introduced for
large-scale immunization in 1959. In 1972, Sabin proposed that WHO should be the custodian of
his poliovirus seed strains. The Director-General of WHO agreed to assume responsibility for
ensuring the proper use of the strains and established a scientific committee, the Consultative
Group on Poliomyelitis Vaccines, to advise WHO on all matters pertaining to their use. Detailed
information on the work of the Consultative Group and the preparation of the seed stocks made
by Behringwerke has been published by Cockburn (35). NRAs should decide on the use of virus
strains and on the detailed procedures applicable to the preparation of virus seed lots for the
production of OPV in their own countries.

¹ https://polioeradication.org/
The original poliovirus seeds produced by Sabin (SO) (25) were sent to Merck which generated seeds from them that were designated as SOM (Sabin Original Merck). Aliquots of SOM were supplied to a number of other manufacturers to enable them to develop their own seeds. Some seed lots were contaminated with SV40 which was present in the primary Rhesus kidney cells, the preferred cell culture system at that time for virus propagation. OPV manufacturers followed various strategies to reduce the contamination, including passage in the presence of specific antibody or treatment with toluidine blue, or thermal inactivation of SV40 in the presence of 1M MgCl₂ that stabilizes poliovirus. In 1974 Behringwerke AG of Marburg/Lahn, Germany, generously agreed to produce SO+1 seeds for WHO free of charge. The Behringwerke type 1 and type 2 seeds have been particularly widely used from the 1970s to date. In the 1950s, it was established that, particularly for the type 3 strain, increase in the passage number correlated with an increase in the reactivity in the MNVT. This finding led to the establishment of rigorous limits on the passage level for vaccine production for all types.

In order to develop a more stable type 3 strain, a new seed was prepared by Pfizer from a single plaque after transfecting susceptible cells with viral RNA extracted from poliovirus at the SO+2 level. This also reduced any residual risk of SV40 contamination. One plaque, which was designated 457-III, was identified with particularly favourable properties (24). Theoretically, vaccine derived from this stock was at passage SO+7. However, the purpose of tracking passage history of seed viruses is to reduce the accumulation of mutations that takes place during their serial propagation. Since plaque purification represents the cloning of a single infectious particle, it eliminates the heterogeneity of viral population, and the passage level is effectively reset to zero. Thus, the cloned stock 457-III was renamed RSO (for RNA-derived Sabin Original). Two additional passages were used to prepare virus master (RSO1) and working seeds (RSO2), and vaccines produced from this virus are at RSO3 level. Retrospectively, the consensus sequence of RSO has been shown to be the same as the consensus of SO (36), but it was more homogeneous and contained lower quantities of viruses with sequence polymorphisms. Consensus sequences of all three Sabin strains are available in GenBank under accession numbers AY184219, AY184220, and AY184221.

The RSO seed was not used for the production of type 3 vaccine until the 1980s when it became clear that the virus stocks passaged from the SOM and other SO+1 seeds were inadequate. Since then, however, it has been widely used by European and American manufacturers as it is of lower virulence in laboratory tests than the SO+1 type 3 seed. The RSO seeds were bought from Pfizer by Sanofi Pasteur (formerly Institut Mérieux, Pasteur Mérieux Connaught and subsequently Aventis Pasteur) which has donated them to WHO.

The virus seeds available from WHO (designated “the WHO master seeds”) are therefore types 1 and 2 at SO+1 level produced by Behringwerke from SO seeds and the type 3 RSO “Pfizer” seed donated by Sanofi Pasteur. The seeds are kept at the National Institute for Biological Standards and Control in the United Kingdom and FDA/CBER in the United States and include a

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proportion of the stocks of the SO+1 seeds formerly held at Istituto Superiore di Sanità in Italy which kindly transferred them (24, 35). The virus seed stocks are available to vaccine manufacturers upon request to the WHO.

In addition to the RSO type 3 seed, a number of manufacturers in China, Japan and Russia have used their own purified seed stocks of Sabin 3 strain that were derived by a combination of passage and plaque purification (cloning). Sequencing of these seed viruses demonstrated that, while they contained low quantities of neurovirulent mutants, there were differences at other genomic sites between these strains and the consensus sequence of Sabin Original virus in the form of SNPs (35). However, there are no reports of any differences in clinical safety or immunogenicity between OPV produced from Pfizer stocks and the alternative seeds of Sabin 3 virus. An overview of virus seeds used in OPV production is given in Appendix 1.

The MNVT, as described in the 1989 requirements (5), has been used as a quality control test and is based on the level and the distribution of virus-specific lesions within the central nervous system produced by vaccine virus upon intraspinal inoculation into the anterior horns of Rhesus or Cynomolgus monkeys as compared to an appropriate reference preparation (37). Because nonhuman primates are used, efforts to complement and eventually replace the test are of considerable importance. WHO has encouraged and supported research on various aspects of poliovirus biology, including the development of alternative animal models, as part of the WHO initiative to promote the development of new norms and standards for vaccines. Two groups of scientists developed transgenic (TgPVR) mice by introducing into the mouse genome the human gene encoding the cellular receptor for poliovirus (38, 39). This receptor, known as CD155, makes TgPVR mice susceptible to poliovirus infection with clinical signs of flaccid paralysis and with histological lesions in the central nervous system similar to those observed in monkeys.

In 1992, WHO initiated a project to evaluate the suitability of transgenic mice for testing the neurovirulence of OPV with the aim of replacing monkeys with mice. The advantages of a neurovirulence test in transgenic mice are:

- a reduction in the number of primates used in quality control of OPV;
- the use of animals of highly-defined genetic and microbiological quality standards;
- a reduction in hazards to laboratory personnel through a reduced need to handle primates;
- a reduction in the time and cost of quality control tests for OPV.

Studies were carried out initially on type 3 monovalent polio vaccines using the TgPVR21 mouse line, generously provided free of charge for the study by the Central Institute for Experimental Animals in Kawasaki, Japan. Researchers at the FDA Centre for Biologics Evaluation and Research (CBER) in Rockville, MD, USA developed an intraspinal inoculation method suitable for tests of vaccine lots. This was evaluated in an international collaborative study on the establishment of a standardized mouse neurovirulence test (TgmNVT) for OPV (40). Several laboratories participated in the collaborative study and results were assessed by WHO at meetings held in 1995, 1997 and 1999 in Geneva, Switzerland, in 1997 in Ottawa, Canada, and in 1998 in Rockville, MD, USA. As a result of these studies, the revised
Recommendations for the production and control of poliomyelitis vaccine (oral) (9) introduced the murine model as an alternative to the MNVT for type 3 poliovirus and further studies demonstrated that this test was also suitable as an alternative to the MNVT for poliovirus types 1 and 2 (12). Laboratories must comply with specifications for containment of the transgenic animals (41). The MNVT and TgmNVT can provide evidence of consistency of production.

The molecular mechanisms and genetic determinants of attenuation and reversion to virulence of all three types of Sabin polioviruses used for the manufacture of OPV have been studied in several laboratories. As discussed above in the context of nOPV, evidence strongly suggests that mutations in the domain V of the internal ribosome entry site (IRES) in the 5’ untranslated region (5’-UTR) of poliovirus genome are critical in determination of the attenuated phenotype (42). A molecular biological test, MAPREC assay, was developed by researchers at CBER to quantify reversion at the molecular level (43). Studies showed that all batches of type 3 OPV contained measurable amounts of revertants with C instead of U at nucleotide 472. Batches that failed the MNVT contained significantly higher quantities of 472-C than batches that passed the test. Studies with coded samples at CBER identified 100% of lots that failed the MNVT (44).

In 1991, WHO initiated a series of international collaborative studies to evaluate MAPREC assay for all three types of polioviruses and to validate appropriate reference materials. Several laboratories participated in the collaborative studies and results were assessed by WHO at meetings held in 1995 and 1997 in Geneva, Switzerland. It was concluded that MAPREC assay was a sensitive, robust, and standardized molecular biological assay suitable for use by manufacturers and NRAs for monitoring the consistency of production of type 3 OPV. The revised Recommendations for the production and control of poliomyelitis vaccine (oral) (9) introduced, for type 3 poliovirus, the use of MAPREC as the in vitro test of preference in place of the rct40 test. Reference materials for MAPREC were established for all three serotypes. For type 3 the International Standard used in MAPREC test defines the threshold of 472-C content above which vaccine lots have a high chance of failing MNVT. Reference materials for comparable positions in type 1 and type 2 are used to provide a measure of production consistency, but they do not define the pass/fail threshold because the amount of domain V mutants that make these vaccine preparations fail MNVT is much higher than their content in production lots.

High Throughput Sequencing (HTS) also known as deep sequencing or next generation sequencing is a powerful methodology that could be applied in many areas of the regulation of biological products. Classical (Sanger) sequencing determines the consensus or average sequence of a population of nucleic acid molecules, whereas HTS determines the sequence of individual molecules in a population. HTS generates multiple reads of each base position and produces large amounts of sequence data very rapidly. Determining the sequence of complete viral genomes is relatively straightforward. While the technology is still evolving rapidly this usually involves amplifying sequences by PCR, using primers which may be either specific for a given sequence or random to pick up any nucleic acid sequence present. HTS could therefore be used in principle to detect adventitious agents whose presence is not even suspected. Given that
HTS determines the sequence of individual molecules it will also detect minority populations and polymorphisms so that revertants can be accurately quantitated. HTS has applications in the quality control of live vaccines and could reduce the need for in vivo testing by demonstrating consistency of production on a previously impossible scale.

The bioinformatic analysis required for HTS is significant and the validation of the method for a specific purpose remains a major issue. It would be possible to determine that the frequency of a particular mutation (single nucleotide polymorphism or SNP) varies from production run to production run. It remains to be determined to what degree this happens and what limits are allowed for the runs to be acceptable. In the context of OPV, HTS could be a replacement for MAPREC when used to monitor the frequency of one or two particular mutations and studies are underway to validate this application. Early evidence showed that HTS can accurately measure the 472-C content of type 3 OPV lots and that it could be an alternative to the MAPREC test (45, 46). Whole genome HTS could be a unique tool in determining product consistency. It has been applied very extensively to nOPV, where it is arguably a more sensitive procedure for monitoring product consistency than animal neurovirulence tests.

Significant developmental work needs to be completed before HTS can be introduced for general regulatory purposes. At its meeting in 2019, ECBS recommended that a study be performed to explore the utility of HTS technology for quality control of OPV made from Sabin strains. The resulting study showed that HTS could accurately quantify 472-C mutants in monovalent bulks of OPV3 and the final product (47). A second phase of this study showed that HTS could also accurately quantify mutations of 480-A/525-C and 481-G for OPV1 and OPV2, respectively (48). The results generated by HTS and MAPREC methods were very well correlated (47-49) so that HTS could in principle be used as an alternative to MAPREC, providing an appropriate test format and analytical process to establish assay validity and pass/fail decisions were agreed with the NRA.

HTS makes it possible to conduct whole-genome sequencing on a routine basis. The degree of sequence heterogeneity expressed in terms of the number of single nucleotide polymorphisms (SNP) at other nucleotide positions in the genome not necessarily linked to any tangible biological properties provides a unique molecular “fingerprint” characterizing a particular virus preparation. HTS is ideally suited for generating quantitative whole-genome SNP profiles of individual vaccine lots that can be used to identify types of polio seed virus and to monitor consistency of manufacture. After appropriate validation and establishing manufacture consistency, quantitative whole-genome SNP profiles of OPV lots it is possible that they could be used for routine lot release instead of MNVT or TgmNVT. If this is the case, appropriate acceptance criteria should be approved by the NRA.

The manufacturer of the final lot must be responsible for ensuring conformity with all the recommendations applicable to the final vaccine (Part A, sections A.5–A.11) even where manufacturing involves only the filling of final containers with vaccine obtained in bulk form from another manufacturing establishment. The manufacturer of the final lot must also be
responsible for any production and control tests performed by an external contract laboratory, if
applicable, with the approval of the NRA.

OPV has been in worldwide use since the 1960s and experience has indicated that human diploid
cells, primary monkey kidney cells, and continuous cell lines derived from them (Vero cells) can
produce safe and effective vaccines.

In 1986, a WHO study group (7) stated that the risks for residual cellular DNA (rcDNA) for
vaccines produced in continuous cell lines should be considered negligible for preparations given
orally. This conclusion was based on the finding that polyoma virus DNA was not infectious
when administered orally (50). For such products, the principal requirement is the elimination of
potentially contaminating viruses. Additional data on the uptake of DNA via the oral route have
been published (51). These studies demonstrated that the efficiency of uptake of DNA introduced
orally was significantly lower than that of DNA introduced intramuscularly. Nevertheless, the
specifics of the manufacturing process and the formulation of a given product should be
considered by NRAs (22) and, where possible, data should be accumulated on the levels of
rcDNA in OPV produced in Vero cells.

International reference materials

WHO International Standards and International Reference Preparations are available to ensure
manufacture and quality control testing of the different versions of OPV meet appropriate
regulatory requirements.

International Standards for the potency testing of tOPV have been available since 1995. More
recently, new International Standards have been established for bOPV, mOPV1, mOPV2 and
mOPV3, with compositions and potencies similar to vaccines needed for the final phase of the
GPEI. Additionally, low titre monovalent type 1, 2 and 3 poliovirus WHO reference reagents are
available for use in reference laboratories to measure the sensitivity of cell cultures for poliovirus
infection.

An International Standard for anti-poliovirus types 1, 2 and 3 antibodies (human) is also available
for the standardization of neutralizing antibody tests for poliovirus.

In addition, International Standards for MAPREC analysis of poliovirus types 1, 2 and 3 (Sabin)
and International Reference Reagents for control of MAPREC assays of poliovirus type 1, 2 and
3 (Sabin) are available. Some of these references might be useful for HTS assays for Sabin OPV
upon suitable validation. Alternatively, new reference materials might be needed for this
purpose.

Reference preparations at the SO+2 passage level, designated WHO/I for type 1 virus, WHO/II
for type 2 virus and WHO/III for type 3 virus, are available upon request through WHO. These
reference preparations are for use in in vivo neurovirulence tests for OPV, both in monkeys and
transgenic mice. The relevant reference materials should be included in each vaccine test (see section A.4.4.7.2). Virus panels for validation and implementation of the transgenic mouse neurovirulence test, as specified in the WHO SOP, are also available.

New non-pathogenic, hyper-attenuated S19 strains of all three serotypes of poliovirus, are available for conducting neutralization assays. S19 strains are polioviruses that replicate in tissue culture but are unlikely to replicate at all in humans should they be exposed even to large amounts and for this reason they can be used outside GAPIII containment requirements.

Some of the references developed for Sabin OPV might be suitable for nOPV assays after suitable validation but establishment of nOPV-specific references might be required.

The above reference materials are available from the National Institute for Biological Standards and Control, Potters Bar, United Kingdom. Full details of these materials, including literature references, are given in Appendix 8.

Part A. Manufacturing recommendations

A.1 Definitions

A.1.1 International name and proper name

The international name should be poliomyelitis vaccine (oral, live, attenuated) with additions to indicate the virus serotype or serotypes of the vaccine and if the vaccine is a novel or Sabin OPV. The proper name should be the equivalent of the international name in the language of the country of origin.

The use of the international name should be limited to vaccines that satisfy the recommendations formulated below.

A.1.2 Descriptive definition

Poliomyelitis vaccine (oral, live, attenuated) is a preparation of live-attenuated poliovirus type 1, 2 or 3 grown in in vitro cultures of suitable cells containing any one type or any combination of the three types of the Sabin strains or novel genetically stabilized attenuated strains, presented in a form suitable for oral administration and satisfying all the recommendations formulated in this document, as applicable.

A.2 General manufacturing recommendations

The general guidance provided in WHO Good manufacturing practices for pharmaceutical products: main principles (52) and WHO Good manufacturing practices for biological products (21) should apply to establishments where OPV is manufactured, with the addition of the following recommendations:

- The production of OPV should be conducted by staff who are healthy and who are examined medically at regular intervals. Steps should be taken to ensure that all

1 https://www.nibsc.org/
persons in the production areas are immune to poliomyelitis. Personnel working in monkey quarters should also be examined for tuberculosis as outlined in Part A, section 2 of Recommendations to assure the quality, safety, and efficacy of BCG vaccine (53).

- The establishment should be in compliance with the current global recommendations for poliovirus containment (15-17, 54).

### A.3 Control of source materials

General production precautions, as formulated in Good manufacturing practices for biological products (21) should apply to the manufacture of OPV, with the additional recommendation that during production only one type of cell should be introduced or handled in the production area at any given time.

#### A.3.1 Cell lines

Some of the licensed OPV products are produced in primary monkey kidney cells (PMKC) (see Part E). However, new OPV manufacturers are encouraged to use cell lines, such as MRC-5 and Vero cells (see section A.3.1), for vaccine production (22).

- **A.3.1.1 Master cell bank (MCB) and working cell bank (WCB)**
  
The use of a cell line for the manufacture of OPVs should be based on the cell bank system. The cell seed and cell banks should conform with the Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (22). The cell bank should be approved by the NRA. The maximum number of passages (or population doublings) allowed between the cell seed, the MCB, the WCB and the production passage level should be established by the manufacturer and approved by the NRA. Additional tests may include but are not limited to: propagation of the MCB or WCB cells to or beyond the maximum in vitro age for production, and examination for the presence of retrovirus and tumorigenicity in an animal test system (22).

- It is important to show that the cell banks (cell seed, MCB and WCB) are free of adventitious agents relevant to the species used in their derivation. Cell banks should be assessed for the absence of adventitious agents that may have been present during production.

  - The WHO Vero reference cell bank 10-87 is considered suitable for use as a cell seed for generating an MCB (22) and is available to manufacturers on application to the Group Lead, Norms and Standards for Biologicals, Technical Specifications and Standards, Department of Health Product Policy and Standards, Access to Medicines and Health Products Division, World Health Organization, Geneva, Switzerland.

- **A.3.1.2 Identity test**
  
Identity tests on the master (MCB) and working cell banks (WCB) should be performed in accordance with WHO’s Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (22) and should be approved by the NRA.
The cell banks should be identified by means of tests such as biochemical tests, immunological tests, cytogenetic marker tests and DNA fingerprinting or sequencing (22). The tests should be approved by the NRA.

A.3.1.3 Cell culture medium

Serum used for the propagation of cells should be tested to demonstrate freedom from bacterial, fungal and mycoplasma contamination by appropriate tests- as specified in Part A, sections 5.2 (55) and 5.3 (56) of the WHO General requirements for the sterility of biological substances - as well as freedom from infectious viruses. Suitable tests for detecting viruses in bovine serum are given in Appendix 1 of the WHO’s Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (22).

Validated molecular tests for bovine viruses may replace the cell culture tests of bovine sera if approved by the NRA. As an additional monitor of quality, sera may be examined for freedom from bacteriophage and endotoxin. Gamma irradiation may be used to inactivate potential contaminant viruses, while recognizing that some viruses are relatively resistant to gamma irradiation.

The source(s) of animal components used in the culture medium should be approved by the NRA. These components should comply with the current WHO guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products (57).

Human serum should not be used. If human serum albumin derived from human plasma is used at any stage of product manufacture, the NRA should be consulted regarding the relevant requirements, as these may differ from country to country. At a minimum, it should meet the WHO Requirements for the collection, processing and quality control of blood, blood components and plasma derivatives (58). In addition, human albumin and materials of animal origin should comply with current WHO guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products (57).

Penicillin and other beta-lactams should not be used at any stage of manufacture, as they are highly sensitizing substances. If well justified, other antibiotics may be used during early stages of production, and should be cleared during the downstream manufacturing process. Clearance should be demonstrated through a residual removal study (or studies) and acceptable residual levels should be approved by the NRA (21).

Nontoxic pH indicators may be added (for example, phenol red at a concentration of 0.002%).

Only substances that have been approved by the NRA may be added.
Bovine or porcine trypsin used for preparing cell cultures should be tested and found to be free of cultivable bacteria, fungi, mycoplasmas and infectious viruses, as appropriate (22). The methods used to ensure this should be approved by the NRA.

In some countries, irradiation is used to inactivate potential contaminant viruses in trypsin. If irradiation is used, it is important to ensure that a reproducible dose is delivered to all batches and to the component units of each batch. The irradiation dose must be low enough so that the biological properties of the reagents are retained while being high enough to reduce viral contamination. Therefore, irradiation cannot be considered a sterilizing process (22). The irradiation method should be validated by the manufacturer and approved by the NRA.

Recombinant trypsin is available and OPV manufacturers are encouraged to use the recombinant trypsin due to reduced risk of contamination compared to animal sourced trypsin; however, it should not be assumed to be free of the risk of contamination and should be subject to the usual considerations for any reagent of biological origin (22).

The source(s) of trypsin of bovine origin, if used, should be approved by the NRA and should comply with the current WHO guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products (57).

A.3.2 Virus seeds

A.3.2.1 Virus strains

Strains of poliovirus used in the production of OPV should be identified by historical records. This should include information on their origin and subsequent manipulation or passage, including steps of recombinant DNA technology used to modify viral genome when applicable.

In addition, it is recommended that the presence of sequence heterogeneities across the entire genome of OPV is determined by HTS and documented as a reference for future characterization of the virus seed lots.

Producers of Sabin OPV can obtain virus master seeds from WHO. Manufacturers receiving this virus master seed may prepare a sub-master seed by a single passage and then prepare their working seed from the characterized sub-master seed. At the time of writing this document the nOPV strains are only available from the developers.

Only virus strains that are approved by the NRA should be used (see General considerations).

A.3.2.2 Virus seed lot system

Vaccine production should be based on the seed lot system. Virus seed lots should not be purified. The virus working seed lot used for the production of vaccine batches should be prepared by a single passage from the virus master seed lot or the virus sub-master seed lot (if used), by a method and at a passage level from the original seed virus approved by the NRA.
Virus master, sub-master and working seed lots should be stored as recommended in WHO Good manufacturing practices for biological products (21), e.g. in temperature-monitored freezers at a temperature of $\leq -60 \, ^\circ\text{C}$ that ensures stability on storage. Guidance on additional characterization of master and sub-master seeds is provided in Part B.

A.3.2.3 Tests on virus master, sub-master and working seed lots

The Sabin virus master seeds provided by WHO are well-characterized, and can be used to prepare sub-master seed using the approved process. All other virus seed lots used for the production of OPV batches, including sub-master seed derived from WHO master seed, should be subjected to the tests listed in this section and should conform to the recommendations set out in Part A, sections A.4.3 (single harvests) and A.4.4.1–A.4.4.4 (monovalent bulks). The control cell cultures for virus seeds production should conform to section A.4.1 (control of cell cultures).

A.3.2.3.1 Tests for adventitious viruses and freedom from detectable SV40 sequences

The virus seed lots should be shown to be free from detectable adventitious viruses and from detectable SV40 DNA when applicable as determined by a validated nucleic acid amplification test. The need for testing SV40 DNA, and other human, simian, bovine or porcine adventitious agents should be based on risk assessment of potential contamination of the cell substrates used to propagate the virus, as well as the adventitious agents that may be inadvertently introduced through the use of raw materials, e.g. animal-derived culture medium components. If necessary, viruses such as bovine polyomavirus, porcine parvovirus or porcine circovirus may be screened for using specific assays, such as molecular assays based on nucleic acid amplification techniques (NAT) (22).

DNA of SV40 is widely used as molecular biological reagent, and contamination of PCR assays is potentially a major problem. One approach is to identify separate genomic regions of SV40 for amplification, and to use one region for screening purposes and the other for the confirmation of repeatedly positive samples. It is useful if the second genomic region used for confirmation varies between isolates from different sources, as it is then possible to show that it has a unique sequence and that positive results are not due to contamination with laboratory strains of SV40. The sensitivity of the PCR assays for the genomic regions used should be established.

New molecular methods with broad detection capabilities are being developed for the detection of adventitious agents. These methods include: (a) degenerate NAT for whole virus families, with analysis of the amplicons by hybridization, sequencing or mass spectrometry; (b) NAT with random primers followed by analysis of the amplicons on large oligonucleotide micro-arrays of conserved viral sequencing, or digital subtraction of expressed sequences; and (c) HTS. These methods might be used in the future to supplement existing methods or as alternative methods to both in vivo and in vitro tests after appropriate validation and with the approval of the NRA (22).

The testing strategy for adventitious virus(es) on seed lots should be based on risk assessment. However, sterility testing for bacteria, fungi and mycoplasmas should be conducted.
A.3.2.3.2 Tests to monitor virus molecular characteristics

A.3.2.3.2.1 Tests in vitro

New virus seed lots used for OPV production should be evaluated for molecular consistency using a suitable test, such as HTS, and should meet the acceptance criteria approved by the NRA. Virus seeds prepared from Sabin strain may be evaluated using MAPREC test and should meet the acceptance criteria described in section A.4.4.7.1. In addition, at least three consecutive monovalent bulks prepared from the new seed virus should meet the acceptance criteria of the applicable in vitro test described in section A.4.4.7.1. Where HTS method is used it should be validated using appropriate standards and materials, and acceptance criteria approved by the NRA. At this point the use of HTS remains a work in progress and is a subject of international collaborative study that may result in the establishment and availability of appropriate reference materials with defined acceptance criteria.

The acceptance criteria for percentage of mutations at positions that are not examined by MAPREC but found to be variable under the conditions used by the manufacture should be based on the molecular characteristics of vaccine batches shown to be safe and immunogenic in clinical studies. The acceptance criteria of HTS should be updated periodically based on manufacturing experience. Acceptance criteria should be approved by the NRA.

nOPV seeds and at least three consecutive monovalent bulks prepared from each new working seed should be characterised by HTS, with particular attention to the regions of the genome that are modified in the parental nOPV strain compared to the Sabin OPV strain. The genetic modifications introduced in domain V of the 5'-untranslated region (UTR) of nOPV include changes in specific base pairs of the hairpin structure where GC and GU pairs were replaced by AU base pairs. Strengthening of the hairpin structure that leads to neurovirulent reversion requires two simultaneous mutations, and the frequency of such double reversions should be minimal. Therefore, HTS analysis should be conducted to ensure that there are no undesirable modifications in the 5'-UTR, with particular attention to changes in base-pairing in domain V.

A.3.2.3.2.2 Neurovirulence tests

A.3.2.3.2.2.1 Neurovirulence tests for virus seeds prepared from Sabin strain

New virus seeds prepared from Sabin strains, except the well characterized WHO master seed, should be evaluated for neurovirulence using MNVT or TgmNVT. Summaries of the MNVT and TgmNVT, including pass/fail criteria, are given in Appendix 2 along with considerations on the choice of assay. The test should be approved by the NRA for the specific product.

The test for neurovirulence in nonhuman primates should be carried out as summarized in Appendix 2 and following the SOP Neurovirulence test of types 1, 2 or 3 live attenuated poliomyelitis vaccines (oral) in monkeys, available from WHO.¹

Under normal circumstances, a new virus working seed will be prepared using the same production protocol and from the same virus master seed or sub-master seed as the currently approved virus working seed. If the TgmNVT has been approved by the NRA for the release of vaccine batches, and if the virus working seed is generated by the same production process, the new seed can be qualified by use of the transgenic mouse test and supporting in vitro data alone. TgmNVT should be carried out as summarized in Appendix 2 and described in detail in the SOP Neurovirulence test of type 1, 2 or 3 live attenuated poliomyelitis vaccines (oral) in transgenic mice susceptible to poliovirus, available from WHO (see above).

In case there are any major changes in the production process for a new virus working seed or virus sub-master seed, full characterization using an in vivo neurovirulence test and HTS will be required (See Part B).

The neurovirulence of the virus working seeds and at least three consecutive monovalent bulks prepared from it should meet the criteria for acceptability given in section A.4.4.7.2 and the appropriate SOP before the virus working seed can be considered suitable for use for the production of OPV, with agreement of the NRA.

A.3.2.3.2.2 Neurovirulence tests for nOPV virus seeds

The virus seed lot used for nOPV production should be evaluated for neurovirulence. The testing strategy (e.g. MVS and/or WVS) and the selection of method (MNVT and/or TgmNVT) should be approved by the NRA. The in vivo neurovirulence test should be carried out as summarized in Appendix 2 and the applicable SOPs available from WHO. The current WHO reference preparation for MNVT derived from Sabin strain is suitable for evaluating neurovirulence of virus seeds and vaccine batches of nOPV.

It is likely that the molecular assays will be more sensitive than the animal tests used to justify the limits chosen. All nOPV producers should generate data in support of replacing in vivo neurovirulence tests with HTS for the evaluation of neurovirulence of nOPV seeds and vaccine batches by examining the entire genome. The acceptance criteria for percentage of mutations should be set in the first instance based on molecular characteristics of vaccine batches shown to be safe in clinical studies that have met the acceptance criteria when tested using an in vivo neurovirulence test. Specifications are likely to change with experience. The data generated will be used to demonstrate consistency and limits should be set on this basis in the longer term.

A.4 Control of vaccine production

Part E contains additional or alternative recommendations for OPV prepared in cultures of primary monkey kidney cells and concerns the testing of the cell substrate used for the production of the vaccine.

A.4.1 Control cell cultures
When human diploid or continuous cell lines are used to prepare cultures for the production of vaccine, a fraction equivalent to at least 5% of the total or 500 ml of cell suspension, or 100 million cells, at the concentration and cell passage level employed for seeding vaccine production cultures, should be used to prepare control cultures. (See Appendix 3 for an example of a flowsheet of tests in cell cultures).

If bioreactor technology is used, the NRA should determine the size and treatment of the cell sample to be examined.

A.4.1.1 Tests of control cell cultures

The treatment of the cells set aside as control material should be similar to that of the production cell cultures, but they should remain uninoculated for use as control cultures for the detection of adventitious agents.

These control cell cultures should be incubated under conditions as similar as possible to the inoculated cultures for at least two weeks and should be tested for the presence of adventitious agents as described below. For the test to be valid, not more than 20% of the control cell cultures should have been discarded for nonspecific, accidental reasons.

At the end of the observation period, the control cell cultures should be examined for evidence of degeneration caused by an adventitious agent. If this examination, or any of the tests specified in this section, shows evidence of the presence of any adventitious agent in the control culture, the poliovirus grown in the corresponding inoculated cultures should not be used for vaccine production.

If not tested immediately, samples should be stored at −60 °C or below.

A.4.1.2 Tests for haemadsorbing viruses

At the end of the observation period, at least 25% of the control cells should be tested for the presence of haemadsorbing viruses using guinea-pig red blood cells. If the latter cells have been stored, the duration of storage should not have exceeded seven days and the storage temperature should have been in the range of 2–8 °C. In tests for haemadsorbing viruses, calcium and magnesium ions should be absent from the medium.

Some NRAs require, as an additional test for haemadsorbing viruses, that other types of red cells, including cells from humans (blood group IV O), monkeys and chickens (or other avian species), should be used in addition to guinea-pig cells.

A reading should be taken after incubation at 2–8 °C for 30 minutes, and again after a further incubation for 30 minutes at 20–25 °C.
If a test with monkey red blood cells is performed, readings should also be taken after a final incubation for 30 minutes at 34–37 °C.

In some countries the sensitivity of each new lot of red blood cells is demonstrated by titration against a haemagglutinin antigen before use in the test for haemadsorbing viruses.

A.4.1.3 Tests for other adventitious agents in cell supernatant fluids
At the end of the observation period, a sample of the pooled supernatant fluid from each group of control cultures should be tested for adventitious agents. For this purpose, 10 ml of each pool should be tested in the same cells, but not the same batch of cells, as those used for the production of vaccine.

A second indicator cell line should be used to test an additional 10 ml sample of each pool. When a human diploid cell line is used for production, a simian kidney cell line should be used as the second indicator cell line. When a simian kidney cell line is used for production, a human diploid cell line should be used as the second indicator cell line (22).

The pooled fluid should be inoculated into culture vessels of these cell cultures in such a way that the dilution of the pooled fluid in the nutrient medium does not exceed 1 part in 4. The area of the cell monolayer should be at least 3 cm² per ml of pooled fluid. At least one culture vessel of each kind of cell culture should remain uninoculated and should serve as a control.

The inoculated cultures should be incubated at a temperature of 35–37 °C and should be observed for a period of at least 14 days.

Some NRAs require that, at the end of this observation period, a subculture is made in the same culture system and observed for at least an additional 14 days. Furthermore, some NRAs require that these cells should be tested for the presence of haemadsorbing viruses.

For the tests to be valid, not more than 20% of the culture vessels should have been discarded for nonspecific, accidental reasons by the end of the test period.

If any cytopathic changes due to adventitious agents occur in any of the cultures, the virus harvests produced from the batch of cells from which the control cells were taken should be discarded.

Some selected viruses may be screened by using specific validated assays which are approved by the NRA, such as molecular techniques (e.g. nucleic acid amplification or HTS) (22).

If these tests are not performed immediately, the samples should be kept at a temperature of −60 °C or below.

A.4.1.4 Identity test
At the production level, the cells should be identified by means of tests approved by the NRA. Suitable methods include, but are not limited to, biochemical tests (e.g. isoenzyme analyses), immunological tests, cytogenetic tests (e.g. for chromosomal markers) and tests for genetic markers (e.g. DNA fingerprinting or sequencing).

**A.4.2 Cell cultures for vaccine production**

**A.4.2.1 Observation of cultures for adventitious agents**

On the day of inoculation with the virus working seed lot, each cell culture or a sample from each culture vessel should be examined visually for degeneration caused by infective agents. If such examination shows evidence of the presence of any adventitious agent the culture should not be used for vaccine production (see section A.4.1.3).

If animal serum is used for cell cultures before the inoculation of virus, the medium should be removed and replaced with serum-free maintenance medium after the cells have been washed with serum-free medium, if appropriate.

**A.4.3 Control of single harvests**

**A.4.3.1 Single harvest**

After inoculation of the production cells with the virus working seed lot, inoculated and control cell cultures should be held at a fixed temperature that is shown to be suitable, e.g. within the range 33–35 °C for Sabin strains.

The range required to produce a consistent satisfactory product for nOPV may be different and should be validated.

The temperature should be controlled within a narrow range (e.g. not vary by more than 0.5 °C from the set temperature). The optimal range for pH, multiplicity of infection, cell density, virus recovery and time of incubation should be established by each manufacturer and should be approved by the NRA.

The incubation time of the viral culture should be established and validated for each OPV product during product development. The virus suspension of Sabin strain should be harvested not later than four days after virus inoculation to limit the number of replication cycles.

Similar appropriate limits should be investigated and set for nOPV harvests.

The inoculated cell cultures should be processed in such a manner that each virus suspension harvested remains identifiable as a single harvest and is kept separate from other harvests until the results of all the tests described in Part A sections A.4.1.2, A.4.1.3, A.4.1.4, A.4.3.3.1, A.4.3.3.2, A.4.3.3.3, A.4.3.3.4 and A.4.3.3.5 have been obtained.

**A.4.3.2 Sampling**
Samples required for the testing of single harvests should be taken immediately on harvesting. If the tests for adventitious agents as described in Part A, section A.4.3.3.3 are not performed immediately, the samples taken for these tests should be kept at a temperature of −60 °C or lower and subjected to no more than one freeze–thaw cycle.

A.4.3.3 Tests on single harvest

A.4.3.3.1 Identity

Each single harvest should be identified using a suitable method, such as an immunological assay on cell culture using specific antibodies or by a molecular method which has been validated and approved by the NRA. If the virus seeds used for production and other poliovirus strains are manipulated or stored at the same production facilities, the identity test should have the ability to distinguish between these strains as well as the ability to distinguish different serotypes of poliovirus.

Neutralization tests can distinguish the serotypes of poliovirus. Molecular method such as sequencing, HTS, or qPCR, can distinguish different strains and serotypes of poliovirus.

Care should be taken to ensure that the sera used are monospecific by titrating them against homotypic and heterotypic viruses of known virus titre. Monoclonal antibodies may be useful in this test.

A.4.3.3.2 Titration for virus content

The virus titre per ml of single harvest should be determined in cell cultures in comparison with an existing reference preparation (see Appendix 4).

A.4.3.3.3 Tests of neutralized single harvests for adventitious agents

For the purposes of the recommendations set out in this section of Part A, the volume of each single harvest taken for neutralization and testing should be at least 10 ml and should be such that a total of at least 50 ml or the equivalent of 500 doses of final vaccine, whichever is the greater, has been withheld from the corresponding single harvest.

The antisera used for neutralization should be of nonhuman origin and should have been prepared in animals other than monkeys, using virus cultured in cells from a species different from that used in the production of the vaccine. Samples of each virus harvest should be tested in human cells and at least one other sensitive cell system.

The neutralized suspensions should be inoculated into bottles of these cell cultures in such a way that the dilution of the suspension in the nutrient medium does not exceed 1 part in 4. The area of the cell sheet should be at least 3 cm² per ml of neutralized suspension. At least one bottle of each kind of cell culture should remain uninoculated and should serve as a control; it should be maintained using nutrient medium containing the same concentration of the specific antiserum used for neutralization.
Animal serum may be used in the propagation of the cells, but the maintenance medium used after inoculation of the test material should contain no added serum other than the poliovirus neutralizing antiserum or foetal calf serum of controlled origin.

The inoculated cultures should be incubated at a temperature of 35-37 °C and should be observed for a period of at least 14 days.

If adequately justified and validated, lower temperatures may be used.

For the tests to be valid, not more than 20% of the culture vessels should have been discarded for nonspecific, accidental reasons by the end of the test period.

If any cytopathic changes due to adventitious agents occur in any of the cultures, the virus harvest should be discarded.

New molecular methods with broad detection capabilities are being developed for detection of adventitious agents. These methods include degenerate NAT for whole virus families with analysis of the amplicons by hybridization, sequencing or mass spectrometry; NAT with random primers followed by analysis of the amplicons on large oligonucleotide micro-arrays of conserved viral sequencing or digital subtraction of expressed sequences; and high throughput sequencing. These methods might be used to supplement existing methods or as alternative methods to both in vivo and in vitro tests after appropriate validation and approval of the NRA (22).

A.4.3.3.4 Sterility tests for bacteria, fungi and mycoplasmas

A volume of at least 10 ml of each single harvest should be tested for bacterial, fungal, and mycoplasmal contamination by appropriate tests, as specified in Part A, sections 5.2 and 5.3 of the WHO General requirements for the sterility of biological substances (55, 56), or by methods approved by the NRA.

Molecular assays, e.g. NAT alone or in combination with cell culture, may be used as an alternative to one or both of the compendial mycoplasma detection methods following suitable validation and agreement from the NRA (22).

A.4.3.3.5 Test for mycobacteria

The virus harvest should be shown to be free from mycobacteria by an appropriate method approved by the NRA.

Molecular assays (e.g. NAT) may be used as an alternative to mycobacteria microbiological culture method tests for the detection of mycobacteria following suitable validation and agreement from the NRA (22).

Some manufacturers test for mycobacteria only at the monovalent bulk stage with the agreement of the NRA.

A.4.3.3.6 Tests for molecular consistency of production
OPV producers may monitor the molecular characteristics of single harvests or monovalent bulks using an in vitro test as described in A.3.2.3.2.1. These data may further demonstrate manufacturing consistency.

A.4.4 Control of monovalent bulk

A.4.4.1 Preparation of monovalent bulk
The monovalent bulk may be prepared by pooling a number of single harvests of the same virus serotype into a single vessel. This bulk should be filtered through a filter that is able to retain cell debris.

The NRA may require further purification of harvests derived from continuous cell lines. If the harvests are derived from human diploid or monkey kidney cells, further purification is not required.

A.4.4.2 Sampling
Samples of the monovalent bulk prepared as described in section A.4.4.1 should be taken immediately and, if not tested immediately, should be kept at a temperature of −60 °C or below until the tests described in the following sections are performed.

A.4.4.3 Identity test
Each monovalent bulk should be identified using a suitable method, as described in Section A.4.3.3.1.

A.4.4.4 Titration for virus content
The virus titre per ml of filtered monovalent bulk should be determined in cell cultures in comparison with an existing reference preparation (see Appendix 4).

The virus titre as determined by this test should be the basis for the quantity of virus used in the neurovirulence tests in monkeys or in transgenic mice (see Part A, section A.4.4.7.2) and for formulation of the final bulk (see Part A, section A.4.5).

The detailed procedures for carrying out this test and for interpreting the results should be approved by the NRA.

A.4.4.5 Sterility tests for bacteria and fungi
The final vaccine bulk should be tested for bacterial and fungal sterility as specified in Part A, section 5.2 of the General requirements for the sterility of biological substances (55).

A.4.4.6 Test for mycobacteria
The virus harvest should be shown to be free from mycobacteria by an appropriate method approved by the NRA.
Molecular assays (e.g. NAT) may be used as an alternative to mycobacteria microbiological culture method tests for the detection of mycobacteria after suitable validation and agreement from NRA (34).

A.4.4.7 Tests to monitor virus molecular characteristics (consistency)
The poliovirus in the filtered monovalent bulk, prepared as described in section A.4.4.1, should be tested in comparison with the seed lot or a reference virus preparation (see Part A, section A.1.3) to ensure that the vaccine virus has not undergone changes during its multiplication in the production cell culture.

A.4.4.7.1 Tests in vitro
The virus in the monovalent bulk should be tested by at least one in vitro test as described in Section A.3.2.3.2.1.

A.4.4.7.1.1 MAPREC
The MAPREC assay is suitable for all three serotypes of Sabin OPV, but not nOPV which should be evaluated for molecular consistency using a suitable test, such as whole genome HTS. Implementation of the assay should be fully validated by each manufacturer and performed according to the WHO SOP Mutant analysis by PCR and restriction enzyme cleavage (MAPREC) for oral poliovirus (Sabin) vaccine, developed from WHO collaborative studies and available from WHO,¹ or according to a validated alternative procedure.

The MAPREC assay should be used to establish the consistency of production once the test has been validated and normal values for the standards have been established. For all Sabin OPV preparations, and depending on a laboratory’s experience with the MAPREC test, an approach using “warning limits” of ±2 standard deviations and “rejection limits” of ±3 standard deviations from the historical mean may be appropriate. Acceptance and rejection criteria should be specific to each manufacturer and each working seed and should be continually updated as each new bulk is prepared. An investigation of consistency should take place if a batch gives results that are inconsistent with previous production batches.

Results should be expressed as ratios relative to the relevant type-specific International Standard for MAPREC analysis of poliovirus (Sabin). The acceptable variation of mutant content from batch to batch should be agreed with the NRA in the light of production and testing experience.

For type 3 (472-C), a batch should be rejected if the level of mutations is above 1.0% when normalized against the International Standard. The limits for types 1 and 2 virus have been less than 2.0% for type 1 Sabin (for the sum of both mutations 480-A, 525-C) and 1.5% for type 2 Sabin (481-G) (59).

If a filtered monovalent bulk fails in a MAPREC assay, it cannot be used in the manufacturing of finished product, and an evaluation of the manufacturing process, including the suitability of the virus working seed, should be undertaken and discussed with the NRA. Filtered monovalent bulks that pass the MAPREC assay should be tested subsequently for in vivo neurovirulence.

The assay for type 3 is highly predictive of in vivo neurovirulence in animal models. No such correlation exists for types 1 and 2 at the level of revertants present in vaccine bulks. For these types the assay results provide a measure of consistency (59).

Non-radioactive methods for MAPREC are available and may be introduced after validation and approval by the NRA.

A.4.4.7.1.2 HTS
Alternative molecular biology methods, such as HTS, that demonstrate an equivalent or better level of discrimination may be used after validation and approval by the NRA. The MAPREC reference materials might be useful for HTS assays for Sabin OPV upon suitable validation. Alternatively, new reference materials might be needed for this purpose.

A.4.4.7.2 Neurovirulence tests
A.4.4.7.2.1 Neurovirulence tests for Sabin OPV
An appropriate in vivo test should be used to evaluate virus monovalent bulks. Summaries of the MNVT and TgmNVT, including pass and fail criteria, are given in Appendix 2 along with considerations on the choice of assay.

The test should be approved by the NRA for the specific product and may use transgenic mice or nonhuman primates or both. The test for neurovirulence in nonhuman primates should be carried out as summarized in Appendix 2 and described in the SOP Neurovirulence test of types 1, 2 or 3 live attenuated poliomyelitis vaccines (oral) in monkeys, available from WHO.

Where the TgmNVT has been approved by the NRA, it should be carried out as summarized in Appendix 2 and described in detail in the SOP Neurovirulence test of type 1, 2 or 3 live attenuated poliomyelitis vaccines (oral) in transgenic mice susceptible to poliovirus, available from WHO (see above). Its use for batch release purposes should follow the appropriate validation and implementation processes according to national and international regulations. This SOP has been validated for vaccines made from Behringwerke SO-derived seeds (types 1 and 2) and RSO-derived seeds (type 3).

To qualify as competent to perform the TgmNVT, there is a requirement for laboratories to complete a standard implementation process as detailed in the WHO SOP. Once qualified as competent, each laboratory should continue to monitor its performance on a routine basis.

The WHO collaborative study demonstrated that MNVT and TgmNVT are equivalent for testing vaccines prepared from RSO seeds but that TgmNVT may fail otherwise acceptable (by MNVT) lots prepared from derivative strains containing additional mutations (40). Therefore, TgmNVT
can be used as a replacement of MNVT for vaccines made from RSO Sabin 3 strain, while the TgmNVT may require further validation for other derivative strains. This may include the development of an appropriate homologous reference.

It is possible that the in vivo neurovirulence test could be omitted in the future when manufacturing consistency has been established based on the results of both in vivo and whole genome HTS. However, additional experience and data are required to establish suitable acceptance criteria for whole genome HTS performed for the control of Sabin OPV.

A.4.7.2.2 Neurovirulence tests for nOPV

Where the results of manufacturing, preclinical and clinical studies have demonstrated the genetic stability of the attenuation to the satisfaction of the NRA, the in vivo MNVT may be omitted for routine manufacturing control of nOPV with the agreement of NRA.

Only monovalent bulks that meet the acceptance criteria using a validated HTS are used to formulate the final product.

The acceptance criteria for percentage of mutations at positions found to be variable under the conditions used by the manufacture should be based on the molecular characteristics of vaccine batches shown to be safe and immunogenic in clinical studies, or vaccine batches that have met the acceptance criteria of an in vivo NVT. When mutations arise at additional positions, a risk assessment should be performed to assess the potential impact on neurovirulence based on current understanding of the genetic basis for the attenuation (60, 61). An in vivo NVT should be performed to assess the suitability of the monovalent bulk when required by the risk assessment. The acceptance criteria of HTS should be updated periodically based on manufacturing experience, and approved by the NRA.

A.4.5 Final bulk

Final bulk may contain one or more serotypes of poliovirus of the same type of strain, Sabin or nOPV. The operations necessary for preparing the final bulk should be conducted in such a manner as to avoid contamination of the product.

The dilution and mixing procedures involved in preparing the final vaccine bulk should be approved by the NRA.

A.4.5.1 Stabilizers

Any stabilizers that may be included in the final bulk should have been shown, to the satisfaction of the NRA, to improve the stability of the vaccine in the concentrations used and not to impair the safety of the vaccine.

All the tests described in Part A, sections A.4.3.3 and A.4.4, should be performed on samples taken before any stabilizers are added where possible.
A.4.5.2 Sterility tests for bacteria and fungi

The final vaccine bulk should be tested for bacterial and fungal sterility, as specified in Part A, section 5.2 of the General requirements for the sterility of biological substances (55).

A.5 Filling and containers

The requirements concerning filling and containers given in WHO good manufacturing practices for pharmaceutical products: main principles (35) and WHO good manufacturing practices for biological products (21) should apply to OPV filled in the final form. Single- and multi-dose containers may be used.

A final filtration may be included just before the filling operations.

The conditions for storage and shipping, as well as the shelf-life should be supported by adequate stability data and approved by the NRA.

A.6 Control tests on the final lot

Samples should be taken from each final lot for the tests described in the following sections. The following tests should be performed on each final lot of vaccine (that is, in the final containers). Unless otherwise justified and authorized, the tests should be performed on labelled containers from each final lot by means of validated methods approved by the NRA. In general, the specification for each test of OPV final lot should be supported by quality attributes of the clinical lots shown to be safe and sufficiently immunogenic in clinical studies and should be approved by the NRA.

A.6.1 Inspection of final containers

Every container in each final lot should be inspected visually or mechanically, and those showing abnormalities should be discarded and recorded for each relevant abnormality.

A.6.1.1 Appearance

The appearance of the vaccine should meet the specification approved by the NRA with respect to its form and colour.

A.6.2 Extractable volume

Unless otherwise justified and authorized, the extractable volume (ml) and the number of drops, using the approved dropper, should be determined in a minimum of five individual final containers, and should meet the specification approved by the NRA.

A.6.3 pH

The pH of the final lot should be tested in a pool of final containers and the result should be within the range shown to be adequate for preserving virus stability.

A.6.4 Identity
An identity test should be performed on at least one labelled container from each final lot using a suitable method as described in Section A.4.3.3.1.

### A.6.5 Sterility tests for bacteria and fungi

Each final lot should be tested for bacterial and fungal sterility, as specified in Part A, section 5.2 of the General requirements for the sterility of biological substances (55), or by methods approved by the NRA.

### A.6.6 Potency

At least three final containers should be selected at random from each final lot and should be individually tested in a single assay. When the vaccine contains more than one poliovirus type, each type should be titrated separately by using appropriate type-specific antiserum to neutralize each of the other types present. The poliovirus content of each serotype, and the total virus content, should be determined. The assay should include a reference preparation as described in Appendix 4 of these recommendations. The minimum virus titre per human dose should be shown to induce adequate immune response in clinical studies.

An upper limit may be established by each manufacturer to ensure lot-to-lot consistency (e.g. based on mean titre $CCID_{50} + 3$ standard deviations). The upper limit should be approved by the NRA.

Based on available data, it is recommended that the estimated mean virus titres for a single human dose of tOPV prepared from Sabin strain should be not less than $10^{6.0}$ $CCID_{50}$ for type 1, $10^{5.0}$ $CCID_{50}$ for type 2, and $10^{5.5}$ $CCID_{50}$ for type 3, as determined in an assay described in Appendix 4. The 95% confidence intervals of the assays should not differ by a factor of more than $0.3 \log_{10}$ of the estimated number of infectious units in the vaccine. Different potency limit may be acceptable if supported by clinical data.

In 1986 the WHO Region of the Americas began to use a trivalent formulation with $10^{5.8}$ $CCID_{50}$ of poliovirus type 3 (62), following a study in Brazil which demonstrated improved immunogenicity when the amount of type 3 virus in the trivalent vaccine was increased (63). The subsequent success in controlling poliomyelitis in the Americas using this formulation led the EPI Global Advisory Group to recommend a formulation of trivalent OPV with $10^{6.0}$, $10^{5.0}$, $10^{5.8}$ $CCID_{50}$ per dose for types 1, 2 and 3 respectively, on a global basis (34, 64).

The potency specifications for nOPV should be set based on the potency of clinical lots shown to induce adequate protective immunity in clinical trials plus human immunogenicity data. An upper limit should also be defined based on available human safety data.

### A.6.7 Thermal stability

Thermal stability should be considered as a vaccine characteristic that provides an indicator of consistency of production. The thermal stability test is not designed to provide a predictive value of real-time stability but to evaluate whether the product complies with a defined specification.
Additional guidance on the evaluation of vaccine stability is provided in WHO’s Guidelines on stability evaluation of vaccines (65).

Three final containers of each final lot should be incubated at 37 °C for 48 hours. The total virus content in both exposed and unexposed containers should be determined concurrently with that of a suitable validated reference preparation. The loss of potency on exposure should be within the limit approved by the NRA.

For trivalent vaccine prepared from Sabin strain, the vaccine passes the test when the loss on exposure is not greater than a factor of 0.5 log_{10} CCID_{50} per human dose. Several OPV manufacturers have recently demonstrated that the thermal stability test specification applied to tOPV formulations (loss on exposure is not greater than a factor of 0.5 log_{10} CCID_{50} per human dose) is not applicable to some monovalent and bivalent OPVs. Some manufacturers have shown that mOPV formulations that failed the current specification of 0.5 log_{10} have an acceptable stability profile throughout the product shelf-life. Therefore, a specification of 0.6 log_{10} has been accepted by the NRAs and by the WHO Prequalification Programme on the basis of documented evidence that the mOPV1 was stable over two years when stored at −20 °C or below and six months when stored at 2–8 °C.

Suitable thermal stability test for nOPV should be established and validated.

A.6.8 Residual antibiotics (if applicable)
If any antibiotics are added during vaccine production, the content of the residual antibiotics should be determined and should be within limits approved by the NRA. This test may be omitted for routine lot release once consistency of production has been established to the satisfaction of the NRA.

A.6.9 Stabilizer (if applicable)
If a stabilizer is added during vaccine production, the content of the stabilizer should be determined and should be within limits approved by the NRA.

A.7 Records
The recommendations given in section 17 of Good manufacturing practices for biological products (21) should apply.

A.8 Retained samples
The requirements given in section 16 of Good manufacturing practices for biological products (21) should apply.

A.9 Labelling
The requirements given in section 14 of Good manufacturing practices for biological products (21) should apply.
The label on the carton, the container or the leaflet accompanying each container should include the following information:

- the designation(s) of the strain(s) of poliovirus contained in the vaccine;
- the minimum amount of virus of each type contained in one recommended human dose;
- the cell substrate used for the preparation of the vaccine, and the nature and amount of any stabilizer present in the vaccine;
- a statement that the vaccine is not to be injected;
- the number of doses in each vial;
- the volume of the dose.

It is desirable for the label to carry the names both of the producer and of the source of the bulk material if the producer of the final vaccine did not prepare it. The nature and amount of the antibiotics present in the vaccine, if any, may be included.

**A.10 Distribution and shipping**

The requirements given in WHO Good manufacturing practices for biological products (21) should apply. Further guidance is provided in WHO’s Model guidance for the storage and transport of time- and temperature-sensitive pharmaceutical products (66).

**A.11 Stability testing, storage and expiry date**

**A.11.1 Stability testing**

Adequate stability studies form an essential part of vaccine development. These studies should follow the general principles outlined in the WHO Guidelines on stability evaluation of vaccines (65) and WHO Guidelines on the stability evaluation of vaccines for use under extended controlled temperature conditions (67). The shelf-life of the final product and the hold time of each process intermediate (such as single harvests, monovalent bulk, final bulk) should be established based on the results of real-time, real-condition stability studies, and approved by the NRA.

The stability of the vaccine in its final container, maintained at the recommended storage temperatures up to the expiry date, should be demonstrated to the satisfaction of the NRA on at least three consecutive lots of final product. Accelerated thermal stability tests may be undertaken to provide additional information on the overall characteristics of the vaccine and may also aid in assessing comparability should the manufacturer decide to change any aspect of manufacturing.

The formulation of the vaccine should be shown to minimize potency loss throughout its shelf-life. In case of potency loss (e.g., when stored at 2-8°C for 6 months), the manufacturer should implement a higher potency limit at release to ensure that all vaccine lots released will meet the minimum potency specification at the end of shelf-life as described in WHO Guidelines on the stability evaluation of vaccines for use under extended controlled temperature conditions (67). Acceptable limits for stability should be agreed with the NRA. Following licensure, ongoing
monitoring of vaccine stability is recommended to support shelf-life specifications and to refine
the stability profile (65). The on-going stability testing programme should be approved by the
NRA and should include an agreed set of stability-indicating parameters, procedures for the
ongoing collection of stability data, and criteria for the rejection of vaccine(s). Data should be
provided to the NRA in accordance with local regulatory requirements.

Where the vaccine is to be stockpiled, manufacturers should conduct real-time stability studies
on monovalent bulks at −40 °C or below, or on finished monovalent, bivalent and trivalent
composition at −20 °C.

Any extension of the shelf-life should be based on stability data and approved by the NRA.

A.11.2 Storage conditions

Before being released by the manufacturing establishment, all vaccines in final containers should
be kept continuously at a temperature that minimizes potency loss, e.g., in the frozen state at a
temperature below −20 °C.

To facilitate vaccine distribution, OPV may be stored at a higher temperature for a specified
period during shipping and distribution in the field, such as at 2–8°C for six months. In addition,
during manufacturing, shipment or in the field, the vaccine may be thawed and refrozen.
Manufacturers should conduct real-time and real-condition stability studies to support the storage
conditions at different temperatures as well as the number of freeze-thaw cycles. The stability
data should demonstrate that the vaccine conforms to the requirements of potency until the
expiry date stated on the label, as approved by the NRA.

A.11.3 Expiry date

The expiry date should be based on the shelf-life as supported by the stability studies and
approved by the NRA. The start of the dating period should be specified, e.g. based on the date
of filling or the date of the first valid potency test on the final lot, and should be approved by the
NRA.

Part B.  Nonclinical evaluation of poliomyelitis vaccines
(oral, live, attenuated)

The nonclinical evaluation of candidate poliomyelitis vaccines (oral, live, attenuated) should be
based on the principles outlined in the WHO guidelines on nonclinical evaluation of vaccines
(19) which provide details on the design, conduct, analysis, and evaluation of nonclinical studies.
In principle, all changes made to a product post-approval should follow the requirements listed in
the WHO general Guidelines (68). The following specific issues should be considered in addition
to the tests described in section A.3.2.3 in the context of a change in virus seed.
B.1 Characterization of a new Sabin virus sub-master seed

In the event that a new Sabin virus sub-master seed is prepared by a single passage from a well characterized master seed, including the WHO master seed, it should be subjected to extensive characterization which should include evaluation of the virus working seeds and at least three monovalent bulks derived from it, as described in section A.4.4.7. Characterization studies must include the evaluation of identity by complete nucleotide sequencing to prove that the new sub-master seed consensus sequence is identical to conventional Sabin master seeds and that the mutational composition (e.g., in MAPREC) is consistent. HTS should be undertaken to evaluate heterogeneity of the virus sequence. These approaches have not yet been formally validated, other than the MAPREC tests for base positions in the 5’ non-coding region of type 3 OPV, as described in section A.4.4.7.1.1. A new virus sub-master seed should be tested for neurovirulence using the MNVT or the TgmNVT, subject to the approval of NRA. Summaries of the MNVT and TgmNVT are given in Appendix 2 along with considerations on the choice of assay.

B.2 Characterization of virus seeds for the production of nOPV

Virus strains used for the production of nOPV were constructed using recombinant DNA technology and are genetically stabilized attenuated strains designed based on current knowledge concerning the molecular mechanisms of attenuation and reversion of poliovirus. The virus master, sub-master (if applicable) and working seed lots used to manufacture a candidate nOPV should be subjected to extensive characterization as described in section A.3.2.3. In addition, genetic stability of the strains used for nOPV production should be confirmed at least at the passage level (or beyond) used to prepare the vaccine using a molecular method approved by the NRA, such as whole-genome HTS analysis.

B.3 Evaluation of immunogenicity of nOPV in suitable models

The genome of nOPV production strains are rationally designed to stabilize the attenuation. However, variation can arise in the viral genome of the nOPV production strains on passage in cell cultures. Whether these genome changes (introduced or cumulated) have any impact on the immunogenicity of the candidate nOPV should be studied using suitable methods, such as the antigenicity and/or the ability to grow in in vitro cell culture of the production strain. If required based on the outcomes of the in vitro testing, transgenic mice with interferon-receptor knock-out and expression of human poliovirus receptor are also available to study vaccine induced neutralizing antibodies. Proof-of-concept nonclinical studies based on type-specific serum neutralizing antibody titres may also assist in the selection of doses to be tested in the dose-finding studies in humans.

Part C. Clinical evaluation of poliomyelitis vaccines (oral, live, attenuated)
Clinical trials should adhere to the principles described in WHO’s Guidelines for good clinical practice (GCP) for trials on pharmaceutical products (68) and Guidelines on clinical evaluation of vaccines: regulatory expectations (20). All clinical trials should be approved by the relevant NRAs.

Some of the issues that are specific to the clinical evaluation of OPVs are discussed in the following sections. These sections should be read in conjunction with the general guidance mentioned above. It is also recommended that manufacturers should consult with relevant NRAs regarding the overall clinical development programme.

Part C considers the provision of clinical data required for:

− nOPV prepared from genetically stabilized attenuated strains;
− new formulations based on licensed OPVs that are derived from Sabin poliovirus strains, including monovalent, bivalent, and trivalent vaccines;
− situations where there have been major changes to the manufacturing process of an established vaccine (e.g., changing from primary monkey kidney cells to a cell line).

Clinical evaluation is not required for a vaccine manufactured using a new virus working seed lot, provided that the passage level is not more than one from the master/sub-master seed lot, the working seed has been characterized, and consistency of the manufacturing process has been demonstrated (see sections A.3.2.3). Generating a new sub-master seed requires extensive characterization but not clinical trials (see Part B).

Vaccine formulations containing one or two poliovirus serotypes have been licensed based on clinical trials in endemic countries. The results of clinical trials in Egypt and northern India have demonstrated that the efficacy of mOPV1 is superior to that of trivalent OPV in terms of inducing immunity against poliovirus type 1 (34, 70). Health authorities have recommended widespread use of this vaccine to eliminate poliovirus type 1 transmission in India. In addition, studies on bOPV containing type 1 and type 3 have demonstrated that bOPV is noninferior to mOPV1 and mOPV3 individually, and superior to tOPV.

C.1 General considerations

The Global Polio Eradication Initiative was prompted by World Health Assembly resolution WHA41.28 in 1988, and has led to a dramatic decrease in poliomyelitis cases globally (26). As a result, efficacy studies for poliovirus vaccines are not feasible, and clinical evaluations and seroprevalence studies should compare the safety and immunogenicity of candidate vaccines with a licensed vaccine (comparator vaccine). The assessment of seroconversion should be based on the elicitation of neutralizing antibodies, which are the basis of protection (26). The approval of a candidate OPV should be based on a clear demonstration of noninferiority compared with a licensed OPV or an OPV used under WHO EUL, as described in section C.2.2 of this document. The relative risk of VAPP for a new candidate vaccine versus approved vaccines cannot be estimated from pre-approval studies but should be addressed as part of post-marketing surveillance. The genetic stability of the novel strain should be monitored.
C.2 Safety and immunogenicity studies

C.2.1 Assessment of the immune response

The presence of neutralizing antibodies against polioviruses is considered a reliable correlate of protection against poliomyelitis. However, immunity induced by one serotype does not provide protection against the other two serotypes. A serum neutralizing antibody titre of $\geq 8$ is considered to be a marker of clinical protection against poliomyelitis (71). The demonstration of an immune response to OPV vaccination should be based on the measurement of neutralizing antibody titres at pre- and post-vaccination time points. Seroconversion for polio antigen is defined as:

- for subjects seronegative at the pre-vaccination time point, postvaccination antibody titres of $\geq 8$;
- for subjects seropositive at the pre-vaccination time point, a $\geq 4$-fold rise in post-vaccination antibody titres. If the pre-vaccination titre is due to maternal antibodies, a 4-fold rise above the expected titre of maternal antibodies based on the pre-vaccination titre declining with a half-life of 28 days indicates seroconversion, or post-vaccination antibody titres of $\geq 8$, whichever is higher.

The assay used to assess serum neutralizing antibodies in the clinical samples should follow the key parameters described in the WHO Manual for the virological investigation of poliomyelitis (72), with the exception of the challenge poliovirus strains. OPV developers are encouraged to use genetically modified poliovirus strains that can be manipulated outside of containment facilities (for example, S19 strains) as challenge viruses. The level of neutralizing antibody present in a serum sample is expressed as a titre, which is the reciprocal of the highest serum dilution that inhibits the viral cytopathic effect in 50% of cell cultures. A reference serum calibrated against or traceable to the appropriate International Standard (see section A.1.3 of this document) should be used to control assay performance.

Geometric mean titres (GMTs), seroconversion rates and reverse cumulative distributions should be provided.

C.2.2 Immunogenicity studies

New candidate OPVs manufactured using different vaccine compositions (e.g. monovalent, bivalent, or trivalent) or from genetically stabilized attenuated strains should be compared with a licensed OPV or an OPV used under WHO EUL. The comparator vaccine(s) selected should have been in use for some years so that some data on effectiveness are available in addition to a reliable description of the safety profile. When no licensed type-matched OPV is available for use in clinical trials, one or more licensed OPV (or nOPV used under WHO EUL) may be used as comparator(s) to cover all serotypes included in the candidate vaccine. For example, a tOPV candidate may be compared to two suitable comparators, one bivalent and the other monovalent, in a noninferiority immunogenicity study. In this case, any potential impact on immunogenicity outcomes (e.g. a negative immune interference) due to different compositions/serotypes between the comparators and candidate vaccines should be considered in study design. Further details on
selection of comparators are described in the WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (20), and the study design should be discussed with and approved by the NRA.

C.2.3 Population
The immunogenicity data provided to support the licensure of a candidate OPV as primary series should include data generated in a naive target population, such as infants. The evaluation of new OPV formulations prepared from Sabin strains may be conducted directly in infants and newborns since safety profiles in these populations have already been established. However, the first clinical study (Phase I) of a candidate nOPV should be performed in healthy adults to assess vaccine safety.

The study exclusion criteria should reflect the current contraindications to administration of OPVs.

C.2.4 Endpoints and analyses
The clinical study protocol should state the primary objective(s) of the study. The neutralizing antibody response to the candidate vaccine should be demonstrated to be non-inferior versus an appropriate licensed OPV, an OPV used under WHO EUL (as described in C.2.2) based primarily on GMTs and/or seroconversion rates. The primary endpoint should be selected according to the study population and the anticipated immune response. For example, very high seroprevalence rates are expected in highly immunized populations, thus having implications for the selection of the non-inferiority margin and therefore the sample size calculation. Further details on demonstrating non-inferiority are described in the WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (20).

Other immunological parameters should be compared in planned secondary analyses (e.g. percentages reaching predefined titres).

C.2.5 Dose-ranging studies
At the time of preparation of this document, all licensed Sabin OPV formulations (e.g. monovalent, bivalent and trivalent) contained the recommended dose for each poliovirus type (not less than $10^{6.0}$ CCID$_{50}$ for type 1, $10^{5.0}$ CCID$_{50}$ for type 2, and $10^{5.5}$ CCID$_{50}$ for type 3). However, development of nOPV or novel formulations with improved stability (addition of stabilizers/excipients) or immunogenicity (used in combination with an adjuvant) may require dose-ranging studies to determine the minimum dose of virus required in CCID$_{50}$ to provide adequate immune responses (20). These data could also be used to support the minimum viral titre that should be present in the vaccine at the end of its shelf-life.

C.2.6 Vaccine virus shedding and transmission
Changes in the viral genome of nOPV candidates or changes in vaccine composition may impact virus replication in the intestinal tract and may influence the ability to induce immune responses and the potential for VAPP or spread to non-target populations. Manufacturers should undertake
studies to determine the profile of the vaccine virus (if applicable, by serotype) excreted in the stools of vaccinees and the duration of shedding. Virus excretion of nOPV candidates or new vaccine formulations should be evaluated alongside a licensed OPV product or an OPV product used under WHO EUL (34). For nOPV, virus recovered from stool samples collected from the vaccinees should be evaluated by HTS to verify the genetic stability of the vaccine candidate. Regions including key attenuating mutations should be examined, but drift in the whole genome should monitored.

C.2.7 Challenge studies with attenuated Sabin poliovirus

Induction of mucosal immunity by the candidate and the comparator vaccines should be determined by the assessment of virus excretion after the administration of a challenge dose of OPV, such as nOPV. Excretion of poliovirus in stool specimens is determined at various intervals immediately before the challenge (day 0) and on days 7, 14, 21 and 28 thereafter (70).

C.2.8 Concomitant administration with other vaccines

An evaluation of the effects of co-administration of an OPV with other vaccines should be considered, taking into account which vaccines are most likely to be given concomitantly in different age groups and populations.

When OPVs are used in an EPI programme simultaneously with other vaccines, it is particularly important that the effects of co-administration should be evaluated (e.g. co-administration studies with rotavirus vaccines which are also administered by the oral route).

Immune responses to all other antigens co-administered with the new OPV should be measured at least in subsets. While the study will usually be powered only to demonstrate non-inferiority with respect to neutralizing antibody against the different poliovirus types used in the vaccine, the protocols should at least include planned secondary analyses of antigen-specific responses. If these analyses indicate that immune responses are lower on co-administration with a new OPV compared to the licensed vaccine(s), NRAs will need to consider the potential clinical consequences on a case-by-case basis.

C.2.9 Pre-licensure safety data

The general approach to the assessment of safety of a new OPV during clinical studies should be in accordance with WHO’s Guidelines on clinical evaluation of vaccines: regulatory expectations (20). Planned safety studies should be supported by a clear scientific rationale. Given the long history of the use of vaccines based on Sabin strains, the NRA may decide that additional pre-licensure safety studies are not required. Where a new vaccine formulation, which has not been used previously, is investigated, larger-scale studies will be needed.

An appropriate pharmacovigilance plan should be developed and approved by the NRA prior to licensure.
C.3  Post-marketing studies and surveillance

Enhanced safety surveillance, particularly for detection of VAPP, should be undertaken during the initial post-approval years in collaboration with NRAs. Environmental surveillance should be conducted. The risk and benefit of using monovalent and bivalent OPV derived from Sabin strains should be carefully considered, as this practice in areas with sub-optimal polio vaccine coverage may lead to the emergence of circulating cVDPVs. Manufacturers and health authorities should work in collaboration with the global polio surveillance laboratory network to monitor new vaccines once they are introduced into immunization programmes. These laboratories have extensive experience in poliovirus surveillance and may provide excellent surveillance and post-marketing support.

The total duration of enhanced surveillance should be regularly reviewed by the NRA. If particular issues arise during pre-licensure studies or during post-licensure safety surveillance, it may be necessary to conduct specific post-licensure safety studies.

Part D. Recommendations for NRAs

D.1  General recommendations

The guidance for NRAs and NCLs given in the WHO Guidelines for national authorities on quality assurance for biological products (73) and Guidelines for independent lot release of vaccines by regulatory authorities (23) should be followed. These guidelines specify that no new biological product should be released until consistency of manufacturing and product quality has been established and demonstrated by the manufacturer.

The detailed production and control procedures, as well as any significant changes in them that may affect the quality, safety and efficacy of live attenuated OPV should be discussed with and approved by the NRA.

For control purposes, the relevant international reference preparations currently in force should be obtained for the purpose of calibrating national, regional and working standards as appropriate (74). The NRA may obtain the product-specific or working reference from the manufacturer to be used for lot release until the international/national standard preparation is established.

Only a monovalent bulk approved by the NRA can be used by the manufacturer for the formulation of a final bulk.

Where the monkey neurovirulence test (MNVT) is performed for the control of the monovalent bulk and the national control laboratory does not perform this test itself, the NCL should carry out a second reading of the histological sections provided by the manufacturer for each monovalent bulk. In addition, the national control laboratory or a contract organization certified by the NRA for proficiency to conduct NVT should perform a second reading of at least four neurovirulence tests on the reference preparations using the monkey neurovirulence test in order to obtain the necessary baseline data for comparison with the neurovirulence of test vaccines.
The national control laboratory should encourage the use of the standard form for the reporting of data on virus activity in the sections taken from histopathological examination.

Where the transgenic mouse neurovirulence test (TgmNVT) is performed for the control of the monovalent bulk and the national control laboratory performs this test itself, it should complete the standard implementation process.

If the national control laboratory does not perform the transgenic mouse neurovirulence test, it should carry out a clinical scoring of mice in parallel with the manufacturer at least at day 3 or day 4. Whether a clinical scoring at day 14 is needed should be justified for each monovalent bulk. Moreover, once a year, the injection of mice should be observed by the national control laboratory. Only appropriately trained staff from a competent national control laboratory can carry out a clinical scoring of mice in parallel with the manufacturer.

In one region of the world, 1 in 10 bulks is also independently tested by a national control laboratory competent in carrying out the test. Other regions that implement the transgenic mouse neurovirulence test may wish to follow this approach.

Consistency of production has been recognized as an essential component in the quality assurance of live attenuated OPV. In particular, the NRA should carefully monitor production records and quality control test results for clinical lots, as well as a series of consecutive lots of the vaccine.

D.2 Official release and certification

A vaccine lot should be released only if it fulfils the national requirements and/or satisfies Part A of these WHO recommendations (23).

A summary protocol for the manufacturing and control of OPV, based on the model provided in Appendix 5, signed by the responsible official of the manufacturing establishment, should be prepared and submitted to the NRA/NCL in support of a request for release of the vaccine for use.

A lot release certificate signed by the appropriate official of the NRA/NCL should then be provided to the manufacturing establishment if requested and should certify that the lot of vaccine in question meets all national requirements, and/or Part A of these WHO recommendations. The certificate should provide sufficient information on the vaccine lot, including the basis of the release decision (by summary protocol review and/or independent laboratory testing). The purpose of this official national lot release process is to assess independently the quality and safety of the batches, to facilitate the exchange of vaccines between countries and should be provided to importers of the vaccines.

A model NRA/NCL lot release certificate for OPV is provided in Appendix 6.
Part E. Recommendations for poliomyelitis vaccines (oral, live, attenuated) prepared in primary monkey kidney cells

The following additional or alternative recommendations are for Sabin OPV prepared in cultures of primary monkey kidney cells and concern the testing of the cell substrate used for the production of the vaccine. They should therefore be added to, or used as an alternative to - the appropriate sections in Part A.4 as follows:

- sections E.1.1.1, E.1.3.1, E.1.4.1 and E.1.4.2 are additions to the corresponding Part A.4 sections as indicated below;
- sections E.1.2.1, E.1.2.2 and E.1.2.3 are replacements for the corresponding Part A.4 sections as indicated below.

All the other recommendations given in Parts A and B of this document are also applicable to this type of vaccine.

E.1 Control of vaccine production

E.1.1 Control of source materials

E.1.1.1 Monkeys used for preparation of kidney-cell cultures and for testing of virus

Addition to section A.4.1.

If vaccine is prepared in monkey kidney-cell cultures, animals should be from a species approved by the NRA, and the animals should be in good health and not previously have been used for experimental purposes.

Manufacturers should use animals from closed or intensively monitored colonies.

The monkeys should be kept in well-constructed and adequately ventilated animal rooms in cages separated in such a way as to prevent cross-infection between cages, together with other adequate precautionary measures. Cage-mates should not be interchanged. The monkeys should be kept in the country of manufacture of the vaccine in quarantine groups1 for a period of not less than six weeks before use. If at any time during the quarantine period the overall death rate of a shipment consisting of one or more groups reaches 5% (excluding deaths from accidents or where the cause was specifically determined not to be an infectious disease), monkeys from that entire shipment should continue in quarantine for a further period of not less than six weeks. The monkeys used should be free of infection. At the end of the extended quarantine period, and following thorough investigations, if any additional monkeys die of the same infectious disease, the entire group is discarded from production.

The groups should be kept continuously in isolation, as in quarantine, even after completion of the quarantine period, until the monkeys are used. After the last monkey of a group has been

1 A quarantine group is a colony of selected healthy monkeys kept in one room, with separated feeding and cleaning facilities, and having no contact with other monkeys during the quarantine period.
taken, the room that housed the group should be thoroughly cleaned and decontaminated before
being used for a fresh group.

In countries in which the kidneys from near-term monkeys are used, the mother should be
quarantined for the term of pregnancy.

All actions taken by working personnel should be based on the assumption that a great potential
hazard exists at all times in the quarantine area. Personnel should be provided with protective
clothing, including gloves, footwear and masks or visors. Street clothes should not be permitted
in the animal rooms. Smoking, eating and drinking should be forbidden while personnel are in
the animal rooms.

A supervisor should be made responsible for reporting unusual illness among employees and for
ensuring that all injuries are properly treated. No worker who has cuts or abrasions on exposed
areas of the body should enter the animal area. Any unexplained febrile illness, even while off
duty, should be considered as potentially related to the employee’s occupation.

Monkeys from which kidneys are to be removed should be anaesthetized and thoroughly
examined, particularly for evidence of tuberculosis and herpes B virus infection.

If a monkey shows any pathological lesion relevant to the use of its kidneys in the preparation of
a seed lot or vaccine, it should not be used, nor should any of the remaining monkeys of the
quarantine group concerned be used unless it is evident that their use will not impair the safety of
the product.

All the operations described in this section should be conducted outside the areas where vaccine
is made.

The monkeys should be shown to be free from antibodies to SV40 virus and simian
immunodeficiency virus.

It is desirable that kidney-cell cultures are derived from monkeys shown to be free from
antibodies to foamy viruses. In some countries, monkeys are tested for antibodies to
herpes B virus.

### E.1.2 Production precautions

The general production precautions called for by the Good manufacturing practices for biological
products (21) should apply to the manufacture of vaccine, with the addition of the following
tests.

- **E.1.2.1 Monkey kidney-cell cultures for vaccine production**

  Replacement of section A.4.2.1 – in conjunction with section E.1.2.2 (below).
Cultures of monkey kidney cells should be prepared from kidneys that have shown no pathological signs. Virus for the preparation of vaccine should be grown by aseptic methods in such cultures. If animal serum is used in the propagation of the cells, the maintenance medium used after virus inoculation should contain no added serum.

To reduce animal use, the virus may be grown in serially passaged monkey kidney-cell cultures from primary monkey kidney cells.

Each group of cell cultures derived from a single monkey, or from no more than 10 near-term monkeys, should be prepared and tested as an individual group.

E.1.2.2 Tests of cell cultures used for vaccine production (see Appendix 7)

Replacement of section A.4.2.1 – in conjunction with section E.1.2.1 (above).

On the day of inoculation with virus working seed lot, each cell culture should be examined for degeneration caused by an infective agent. If, in this examination, evidence is found of the presence in a cell culture of any adventitious agent, the entire group of cultures concerned should not be used for vaccine production.

On the day of inoculation with the virus working seed lot, a sample of at least 30 ml of the pooled fluid removed from the cell cultures of the kidneys of each single monkey, or from no more than 10 near-term monkeys, should be divided into two equal portions. One portion of the pooled fluid should be tested in monkey kidney-cell cultures prepared from the same species, but not the same animal, as that used for vaccine production. The other portion of the pooled fluid should be tested in kidney-cell cultures from another species of monkey, provided that the tests are done in cell cultures from at least one species known to be sensitive to SV40 virus. The pooled fluid should be inoculated into bottles of these cell cultures in such a way that the dilution of the pooled fluid in the nutrient medium does not exceed 1 part in 4. The area of the cell sheet should be at least 3 cm² per ml of pooled fluid. At least one bottle of each kind of cell culture should remain uninoculated and should serve as a control.

When the monkey species used for vaccine production is known to be sensitive to SV40 virus, a test in a second species may be omitted with the approval of the NRA.

Animal serum may be used in the propagation of the cells, provided that it does not contain SV40 antibody or other inhibitors, but the maintenance medium used after inoculation of the test material should contain no added serum except as described below.

The cultures should be incubated at a temperature of 35–37 °C and should be observed for a total period of at least four weeks. During this observation period, and after not less than two weeks’ incubation, from each of these cultures at least one subculture of fluid should be made in the same tissue culture system. The subculture should also be observed for at least two weeks.
Serum may be added to the original culture at the time of subculturing, provided that the serum does not contain SV40 antibody or other inhibitors. Immunochemical techniques may be useful for detecting SV40 and other viruses in the cells.

A further sample of at least 10 ml of the pooled fluid should be tested for the presence of herpes B virus and other viruses in rabbit kidney-cell cultures. Serum used in the nutrient medium of these cultures should have been shown to be free from inhibitors. The sample should be inoculated into bottles of these cell cultures in such a way that the dilution of the pooled fluid in the nutrient medium does not exceed 1 part in 4. The area of the cell sheet should be at least 3 cm\(^2\) per ml of pooled fluid. At least one bottle of the cell cultures should remain uninoculated and should serve as a control.

The cultures should be incubated at a temperature of 35–37 °C and should be observed for a period of at least two weeks.

It is suggested that, in addition to these tests, a further sample of 10 ml of pooled fluid removed from the cell cultures on the day of inoculation with the seed lot virus should be tested for the presence of adventitious agents by inoculation into cell cultures sensitive to measles virus.

For the tests to be valid, not more than 20% of the culture vessels should have been discarded for nonspecific, accidental reasons by the end of the respective test periods.

If, in these tests, evidence is found of the presence of an adventitious agent, the single harvest from the whole group of cell cultures concerned should not be used for vaccine production.

If the presence of the herpes B virus is demonstrated, the manufacture of vaccine should be discontinued and the NRA should be informed. Manufacturing should not be resumed until a thorough investigation has been completed and precautions have been taken against any reappearance of the infection, and then only with the approval of the NRA.

If these tests are not carried out immediately, the samples of pooled cell-culture fluid should be kept at a temperature of −60 °C or below, with the exception of the sample for the test for B virus, which may be held at 4 °C provided that the test is done not more than seven days after the sample has been taken.

E.1.2.3 Test of control cell cultures

Replacement of section A.4.1.

Cultures prepared on the day of inoculation with the virus working seed lot from 25%, but not more than 2.5 litres, of the cell suspension obtained from the kidneys of each single monkey, or

\(^1\) Human herpesvirus (herpes simplex) has been used as an indicator for freedom from B virus inhibitors because of the danger of handling herpes B virus.
from not more than 10 near-term monkeys, should remain uninoculated and should serve as
controls. These control cell cultures should be incubated under the same conditions as the
inoculated cultures for at least two weeks, and should be examined during this period for
evidence of cytopathic changes. For the tests to be valid, not more than 20% of the control cell
cultures should have been discarded for nonspecific, accidental reasons. At the end of the
observation period, the control cell cultures should be examined for degeneration caused by an
infectious agent. If this examination, or any of the tests required in this section, shows evidence
of the presence in a control culture of any adventitious agent, the poliovirus grown in the
corresponding inoculated cultures from the same group should not be used for vaccine
production.

E.1.2.3.1 Tests for haemadsorbing viruses
At the time of harvest, or not more than four days after the day of inoculation of the production
cultures with the virus working seed lot, a sample of 4% of the control cell cultures should be
taken and should be tested for haemadsorbing viruses. At the end of the observation period, the
remaining control cell cultures should be similarly tested. The tests should be carried out as
described in Part A, section A.4.1.2.

E.1.2.3.2 Tests for other adventitious agents
At the time of harvest, or no more than seven days after the day of inoculation of the production
cultures with the virus working seed lot, a sample of at least 20 ml of the pooled fluid from each
group of control cultures should be taken and tested in two kinds of monkey kidney-cell culture,
as described in Part E, section E.1.2.2.

At the end of the observation period for the original control cell cultures, similar samples of the
pooled fluid should be taken and the tests referred to in this section in the two kinds of monkey
kidney-cell culture and in the rabbit-cell culture should be repeated, as described in Part E,
section E.1.2.2.

If the presence of herpes B virus is demonstrated, the production cell cultures should not be used
and the measures concerning vaccine production described in Part E, section E.1.2.2, should be
taken.

In some countries, fluids are collected from the control cell cultures at the time of virus
harvest and at the end of the observation period. Such fluids may then be pooled before
testing for adventitious agents.

E.1.3 Control of single harvests
Addition to section A.4.3.

E.1.3.1 Tests for neutralized single harvests in monkey kidney-cell cultures
A sample of at least 10 ml of each single harvest should be neutralized by type-specific
poliomyelitis antiserum prepared in animals other than monkeys. In preparing antisera for this
purpose, the immunizing antigens used should be prepared in non-simian cells.
Care should be taken to ensure that the antiserum used is monospecific. This may be demonstrated by titration of the antiserum against homotypic and heterotypic virus of known virus titre using the same dilution of the antiserum as that used for neutralization.

Half (corresponding to at least 5 ml of single harvest) of the neutralized suspension should be tested in monkey kidney-cell cultures prepared from the same species, but not the same animal, as that used for vaccine production. The other half of the neutralized suspension should be tested in monkey kidney-cell cultures from another species, provided that the tests are done in cell cultures from at least one species known to be sensitive to SV40 virus.

The neutralized suspensions should be inoculated into bottles of these cell cultures in such a way that the dilution of the suspension in the nutrient medium does not exceed 1 part in 4. The area of the cell sheet should be at least 3 cm² per ml of neutralized suspension. At least one bottle of each kind of cell culture should remain uninoculated to serve as a control and should be maintained using nutrient medium containing the same concentration of the specific antiserum used for neutralization.

Animal serum may be used in the propagation of the cells provided that it does not contain inhibitors, but the maintenance medium used after the inoculation of the test material should contain no added serum other than the poliovirus neutralizing antiserum, except as described below.

The cultures should be incubated at a temperature of 35–37 °C and should be observed for a total period of at least four weeks. During this observation period, and after no less than two weeks’ incubation, at least one subculture of fluid should be made from each of these cultures in the same tissue culture system. The subcultures should also be observed for at least two weeks.

Serum may be added to the original cultures at the time of subculturing provided that the serum does not contain inhibitors. Immunohistochemical techniques may be useful for detecting SV40 and other viruses in the cells.

It is suggested that, in addition to these tests, a further sample of the neutralized single harvest is tested by inoculation of 10 ml into human cell cultures sensitive to measles virus.

For the tests to be valid, not more than 20% of the culture vessels should have been discarded for nonspecific, accidental reasons by the end of the respective test periods.

If any cytopathic changes occur in any of the cultures, the causes of these changes should be investigated. If the cytopathic changes are shown to be due to unneutralized poliovirus, the test should be repeated. If there is evidence of the presence of SV40 virus or other adventitious agents attributable to the single harvest, that single harvest should not be used for vaccine production.
E.1.4 Control of monovalent bulk

Addition to section A.4.

E.1.4.1 Monovalent bulk (before filtration)

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 in at least 10 healthy rabbits each weighing between 1.5 and 2.5 kg. The sample should consist of at least 100 ml. Each rabbit should receive not less than 10 ml and not more than 20 ml, of which 1 ml is given intradermally at multiple sites, and the remainder subcutaneously. The rabbits should be observed for between three and five weeks for death or signs of illness.

It is suggested that the sample should consist of at least 1% of monovalent bulk, provided that this is not less than 100 ml, up to a maximum of 500 ml.

All rabbits that die after the first 24 hours of the test should be examined by autopsy, the brain and organs being removed for detailed examination to establish the cause of death. Animals showing signs of illness should be humanely killed and subjected to a similar autopsy.

The monovalent bulk passes the test if no more than 20% of the inoculated rabbits show signs of intercurrent infection during the observation period and if none of the rabbits shows evidence of infection with B virus or other adventitious agents or lesions of any kind attributable to the bulk suspension.

If the presence of B virus is demonstrated, the measures concerning vaccine production described in Part E, section E.1.2.2, should be taken.

A test for the presence of Marburg virus may be carried out in guinea pigs.

E.1.4.2 Monovalent bulk (after filtration)- tests for retroviruses

Test samples from the filtered monovalent bulk should be examined for the presence of retroviruses by an assay for reverse transcriptase (RTase) acceptable to the NRA.

Authors and acknowledgements

The first draft of this document was prepared by the WHO drafting Group composed of: Dr K. Chumakov, United States Food and Drug Administration, the USA; Dr C. Li, National Institutes for Food and Drug Control, P.R. China; Dr L. Mallet, European Directorate for the Quality of Medicines & HealthCare, Council of Europe, France; Dr J. Martin, National Institute for Biological Standards and Control, the United Kingdom; Dr T. Wu, Health Canada, Canada; and Dr T.Q. Zhou, Technologies, Standards and Norms, World Health Organization, Switzerland.

Acknowledgement is also due to Dr P. Minor, Consultant, UK, for conducting critical review and providing valuable input.
The first draft was then reviewed at a WHO informal consultation on revision of recommendations to assure the quality, safety and efficacy of oral poliovirus vaccines, held virtually on 15-17 November 2021, and attended by: Miss P. Agsiri, Institute of Biological Products, Department of Medical Sciences, Ministry of Public Health, Thailand; Dr L. R. Andalucia, Drug, Narcotic, Psychotropic, Precursor and Addictive Substance Standardization, The Indonesian Food and Drug Authority, Indonesia; Dr M. Arita, National Institute of Infectious Diseases, Japan; Dr A. Bhardwaj, Central Drugs Standard Control Organisation, India; Mr R. M. Bretas, Agência Nacional de Vigilância Sanitária, Brazilian Health Regulatory Agency, Brazil; Dr M. Bruysters, National Institute for Public Health and the Environment, Centre for Health Protection, The Netherlands; Dr K. Chumakov, United States Food and Drug Administration, the USA; Ms G. Cooper, National Institute for Biological Standards and Control, Centre of the Medicines and Healthcare Products Regulatory Agency, UK; Dr V. Dhawan, Ministry of Health and Family Welfare, India; Dr S. Fakhrzadeh, Food and Drug Administration, Ministry of Health and Medical Education, Islamic Republic of Iran; Dr E. Grabski, Paul-Ehrlich-Institut, Federal Institute for Vaccines and Biomedicines, Germany; Dr N. Gupta, Indian Council of Medical Research, Department of Health Research, Ministry of Health & Family Welfare, India; Dr P. Haldar, Government of India, India; Mrs T. JivapaiaSarmpong, King Mongkut's University of Technology Thonburi (Bangkhuntian), Thailand; Dr J. Konz, Center for Vaccine Innovation and Access, PATH, USA; Dr C. Li, National Institutes for Food and Drug Control, P.R. China; Dr K. Mahmood, Vaccine Development Global Program, PATH, USA; Dr M. Majumdar, National Institute for Biological Standards and Control, Centre of the Medicines and Healthcare Products Regulatory Agency, UK; Dr L. Mallet, European Directorate for the Quality of Medicines & Healthcare, Council of Europe, France; Dr J. Martin, National Institute for Biological Standards and Control, the United Kingdom; Dr C. Milne, European Directorate for the Quality of Medicines & Healthcare, Council of Europe, France; Dr P. Minor, consultant, United Kingdom; Sr Si Normasari, National Quality Control Laboratory Development of Drug and Food, Indonesian Food and Drug Authority, Indonesia; Dr V. Pithon, Agence nationale de sécurité du médicament et des produits de santé (French National Agency for Medicines and Health Products Safety), France; Dr D. E. Putri, Biological Product Registration, Indonesian Food and Drug Authority, Indonesia; Dr S. E. Reddy, Central Drugs Standard Control Organisation, India; Dr L. Stephens, National Institute for Biological Standards and Control, Centre of the Medicines and Healthcare Products Regulatory Agency, UK; Dr A. Macadam, National Institute for Biological Standards and Control, Centre of the Medicines and Healthcare Products Regulatory Agency, UK; Dr S. Pumiamorn, Institute of Biological Products, Department of Medical Sciences, Ministry of Public Health, Thailand; Dr G. Waeterloos, Sciensano, Belgium; Dr T. Wu, Health Canada, Canada. Representatives of Developing Countries Vaccine Manufacturers Network (DCVMN): Dr R. Cuber, Bio-Manguinhos, Brazil; Dr R. Dhare, Serum Institute of India, India; Ms T. T. B. Hanh, Polivac, Vietnam; Mr W. Hidayat, BioFarma, Indonesia; Mr L. Q. Hung, Polivac, Vietnam; Dr M. Jain, Panacea Biotec, India; Mr S. Kosaraju, Biological E, India; Dr Y. Li, Institute for Medical Biology of Chinese Academy of Medical Sciences, People's Republic of China; Dr K. Maithal, Zydus-Cadila Healthcare, India; Dr S. B. Rajan, Zydus-Cadila Healthcare, India; Mr M. I. Masud, Incepta Vaccine, Bangladesh; Dr L. Mohanty, Panacea Biotec, India; Mr P. Morgon, Cansino Bio,
Based on the outcomes of the above informal consultation, the second draft was prepared by the above WHO drafting group with critical input from Dr P. Minor, Consultant, UK. The resulting draft document was then posted on the WHO Biologicals website during February and March 2022 for a first round of public consultation. Feedback and comments were received from: Dr J. Konz, Dr C. Gast, and Dr M. Toher, PATH, USA; Dr M. Ergasheva, «State Centre of Expertise and Standardization of Medicines, Medical Devices and Medical Equipment», Ministry of Health of the Republic of Uzbekistan, Uzbekistan; Dr R-C Guimarães, Bio-Manguinhos / Fiocruz; Dr I. Fradi, Ministry of Health, Tunisia; Dr C. Milne, EDQM, France; Dr V. Pithon, ANSM, France; Dr D. Das and Dr F. Atouf, United States Pharmacopeia, USA; Dr A.E. de Almeida, FIOCRUZ-Oswaldo Cruz Foundation, INCQS-National Institute for Quality Control in Health, Brazil; Dr A. Malkin, Chumakov Federal Scientific Center for Research & Development of Immune-and-Biological Products of the Russian Academy of Sciences, Russia.

Taking into consideration the comments received, the document WHO/BS/2022.2423 was prepared by the WHO drafting Group: Dr K. Chumakov, United States Food and Drug Administration, the USA; Dr C. Li, National Institutes for Food and Drug Control, P.R. China; Dr L. Mallet, European Directorate for the Quality of Medicines & HealthCare, Council of Europe, France; Dr J. Martin, National Institute for Biological Standards and Control, the United Kingdom; Dr T. Wu, Health Canada, Canada; and Dr T.Q. Zhou, Technologies, Standards and Norms, World Health Organization, Switzerland.
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Appendix 1

Overview of virus seeds used in OPV production

The history of the poliovirus strains used in the production of OPV is well documented (1, 2, 3). This appendix gives an overview of virus seeds used in OPV production.

The flow diagrams in Figures 1 and 2 show the history of seed virus and reference materials used in manufacture of OPV from Sabin 1 and Sabin 2 (Figure 1) and Sabin 3 (Figure 2) strains. Concentric circles indicate progressive virus passages made to prepare master seed stocks, working seed stocks and production lots of vaccine. Where relevant, sub-master seed stocks are identified in the footnotes. Different seed viruses are identified as SO (Sabin Original), SOM (Merck stock of SO), SOB (Behringwerke stock of SO), Pfizer (otherwise known as re-derived SO, or RSO), SOJ (Japanese stock of SO) and SOR (Russian stock of SO).

These figures provide only a historical overview of the use of different seeds derived from the Sabin vaccine strain in OPV production. They do not indicate any WHO "qualification" or "approval" of the strains or vaccines in the context of this document.

The origin of the nOPV given Emergency Use Listing by WHO has been published (4) but it is not yet supplied as a seed by WHO. The design, modifications and their purposes are described in Figure 3.
Figure 1. Types 1 and 2 OPV
Figure 2. Type 3 OPV

Schematic of the nOPV2 genome showing modifications and their locations. The sequence of 5'UTR domain V (S15 domV) prevents an increase in domV thermostability by single point mutations; to prevent replacement of domV attenuation elements by recombination, the cre element, essential for poliovirus replication, was relocated from its original position in the 2C coding region to the 5'UTR (5' cre5). The original cre was inactivated by mutations (cremut); 3Dpol mutations HiFi (D53N) and Rec1 (K38R) reduce overall virus adaptation capacity by reducing mutation and recombination rates, respectively.
Figure 3. nOPV2 Vaccine Design
Names of manufacturers shown on Figures 1 and 2 are as follows:

Belgium GlaxoSmithKline Biologicals
China (1) Institute of Medical Biology, Kunming
China (2) China National Biotec Group, Beijing Tiantan Biological Products Company
France Sanofi Aventis
Indonesia PT Bio Farma
Iran Razi Vaccine and Serum Research Institute
Italy Novartis Vaccines
Japan Japan Poliomyelitis Research Institute
Mexico Biologics and Reagents Laboratories of Mexico
Russia Chumakov Federal Scientific Center for Research & Development of Immune-
and-Biological Products of Russian Academy of Sciences
Serbia Torlak Institute of Virology, Vaccines and Serum
Viet Nam Center for Research and Production of Vaccines and Biologicals

Notes:
2. WHO master seed stock.
4. Type 1 seed stock prepared at JPRI by four passages of SOM, including three terminal
dilution passages (passage level SO+5). Type 2 seed stock prepared at JPRI by one passage
of SOM (SO+2).
5. Seed stock prepared at JPRI by one passage of SOB (SO+2).
6. Novartis performed an additional passage to prepare sub-master seed stock from which a
working seed was produced.
7. Six plaques were selected, pooled together, and grown to produce seed stock in Russia.
10. Produced by JPRI in 1969 from SO stock by one passage (SO+1).
11. Prepared from SOJ by passages in AGMK cells (SOJ+9), including two plaque purifications
and three terminal passages (SO+10).
12. Prepared from SOJ by passages in AGMK cells (SOJ+6), including two plaque purifications
(SO+7).

References
2. Sabin AB, Boulger L. History of Sabin attenuated poliovirus oral live vaccine strains.

In vivo tests for neurovirulence, and considerations in relation to assay choice

Live attenuated vaccines were developed by Sabin in large part by use of nonhuman primates, particularly old world monkeys for measuring the level of residual neurovirulence. In the 1980s, tests of vaccine bulks and seeds were standardized as a single dose of test material given by intraspinal inoculation tested concurrently with a homologous reference. Vaccines derived from the Sabin strains that pass the monkey neurovirulence test (MNVT) have been shown to have an acceptable safety profile. However, in its current form, the MNVT is regarded as a test of consistency and it is not known whether vaccines that fail the test are virulent in human recipients. Tests designed to replace the MNVT should be able to detect the same changes from batch to batch with similar sensitivity. A neurovirulence test in transgenic mice (TgmNVT) expressing the human polio virus receptor (TgPVR21 mice) has been developed as an alternative to the MNVT for all three poliovirus serotypes.

Summaries of the MNVT and TgmNVT for Sabin OPV are given below, along with the implementation process for the TgmNVT. It is assumed that the in vivo neurovirulence test procedures and acceptance criteria applied to Sabin OPV are suitable for the evaluation of nOPV.

1. Summary of the MNVT

1.1 Key features

A detailed set of standard operating procedures for the “Neurovirulence test of types 1, 2 or 3 live attenuated poliomyelitis vaccines (oral) in monkey” is available from WHO.\textsuperscript{1} Between 5.5 and 6.5 log_{10} CCID_{50} of monovalent virus is delivered in a single dose by intraspinal inoculation into the lumbar cord. A back titration of the inoculum should be carried out after the inoculation step is completed. Residual paralysis, if any, is noted over the following 17–22 days. The animals are sacrificed at the end of the test or earlier on humane grounds and prepared for histological examination of the central nervous system. Regions are scored for damage on a scale from 1 to 4, and a mean lesion score is calculated for each monkey and then for all the monkeys in the test. The clinical signs do not form part of the assessment or of the pass/fail criteria. The homologous WHO/SO+2 reference is tested in parallel. For a new laboratory, the implementation process should be agreed with the NRA.

1.2 Number of animals

The number of monkeys has been chosen on statistical grounds, considering the variability of the test, such that a satisfactory vaccine will only twice give the lesion score of a reference preparation in 1% of tests and therefore be incorrectly scored as a fail. Valid animals must show some sign of histological damage as evidence of correct placement of active virus. The number of valid monkeys required per virus preparation is 11 for types 1 and 2 and 18 for type 3.

\textsuperscript{1}Available from: https://www.who.int/teams/health-policy-and-standards/standards-and-specifications/vaccine-standardization/poliomyelitis.
Because a reference must be tested at the same time, the total number of monkeys is at least 22 for types 1 and 2 and 36 for type 3.

1.3 Sections examined

Sections are examined from defined regions of the spinal cord and brain and scored histologically for virus activity on a scale of 1 (cellular infiltration only) to 4 (massive neuronal damage). At least 29 sections are examined per monkey, as specified in the WHO standard operating procedures for the MNVT. The readings are used to generate the mean lesion score for the animal, and the mean lesions scores for all animals are then used to generate the mean lesion score for the test as a whole.

1.4 Pass/fail criteria

The pass/fail criteria are based on the variation in the test from run to run, established from the scores obtained with the reference preparation and specific to each laboratory and operator. The within-test variance is used to calculate the statistical constants C₁, C₂ and C₃. If the mean lesion score of the test vaccine is greater than that of the concurrently tested reference by more than C₁, the vaccine is not acceptable. If the test vaccine gives a higher score than the reference but the difference in scores lies between C₁ and C₂, the vaccine may be retested and the results pooled; if the difference for the pooled test results is greater than C₃, the vaccine fails.

The values for C₁, C₂ and C₃ are initially established on the basis of the data accumulated after four qualifying tests. These values should then be updated after every test until nine tests have been performed. After that, the C values are based on the last 10 tests performed. The C values must be established for each testing laboratory.

2. Summary of the TgmNVT

2.1 Key features

The detailed operating procedures for the TgmNVT, "WHO neurovirulence test of type 1, 2 or 3 live attenuated poliomyelitis vaccines (oral) in transgenic mice susceptible to poliovirus", are available from WHO.¹ The test for neurovirulence of polio vaccines in transgenic mice involves the intraspinal inoculation of a defined strain of transgenic mice carrying the human receptor for poliovirus with small volumes of the test vaccine. Two virus concentrations are used and the read-out of the test is based on the clinical dose response. A reference preparation is tested at the same time and a clearly defined process has been established for implementation of the test in a new laboratory.

2.2 Strain of transgenic mouse

Different transgenic mouse lines differ in their sensitivity to polio infection depending on the particular transgenic construct and the genetic background, and only strains from a source approved by WHO should be used. Currently the only approved transgenic mouse strain is

TgPVR21, developed in Japan and sourced from the developers or from an approved subcontractor.

2.3 Titration of virus

Two doses of virus are inoculated in a volume of five microlitres: for type 1, 1.75 and 2.75 CCID$_{50}$; for type 2, 5.0 and 6.0 CCID$_{50}$; and for type 3, 3.5 and 4.5 CCID$_{50}$. The inocula must be prepared and titrated accurately to ensure that these doses are given; the precision of the determinations should be better than $+/-$ 0.3 log$_{10}$. A back titration of the inoculum should be performed after the inoculation step is completed.

2.4 Inoculation and observation of animals

Animals procured at age 5−6 weeks are randomized to cages and allowed to recover for at least seven days. They are then appropriately anaesthetized and inoculated with five microlitres of diluted test virus between the last thoracic and first lumbar vertebrae. Animals are observed for clinical signs once a day for the next 14 days and ultimately scored either as normal (slight weakness or no signs) throughout or paralysed (paresis on two consecutive days or paralysis on a single day). The lower and higher doses of the reference should give more than 5% and less than 95% of animals paralysed, respectively, for the test to be valid. A test requires 128 mice for one vaccine plus the reference tested concurrently, or 192 for two vaccines and the reference. The reference is the same as that used in the monkey test; the use of other references may be acceptable but should be validated.

The vaccine passes if it is not significantly more virulent than the reference defined in terms of the log odds ratio and statistical constants L1 and L2 which are based on the reproducibility of the test and define the pass/fail criteria and the grey zone in which a retest is required. The acceptance and rejection limits, L1 and L2, were selected so that a test vaccine which is equivalent to the reference will have a 0.95 probability of passing and a 0.01 probability of failing, respectively. The constants are regularly updated. Statistical evaluation of test validity includes linearity and dose and gender effects.

3. Implementation process of the TgmNVT

If a manufacturer wishes to use the transgenic mouse test, relevant validation data should be available for their specific product to demonstrate the test’s applicability. This may include reference to the extensive collaborative studies by which the test was originally developed. A clear stepwise process for implementing the TgmNVT has been established; it involves training in the inoculation technique through injection of Indian ink, tests with vaccines, and testing of a blinded evaluation panel containing vaccines that pass, fail or marginally fail the test. Competence in clinical scoring is acquired through a standardized training procedure which involves parallel scoring with an experienced scorer and criteria for declaring a trainee competent.

Testing should be performed according to procedures specified in the WHO standard operating procedures for the TgmNVT, using appropriate WHO reference materials unless modified
procedures have been validated and shown to be suitable. The test chosen should be used to test
virus seeds and bulks, as described in section A.4.4.7.2.

4. Considerations for the choice of assay for the evaluation of Sabin OPV

The following specific issues suggest that care should be taken in the selection of the in vivo
tests to be performed for neurovirulence and that the selection should be justified. The report of
the WHO Working Group Meeting to Discuss the Revision of the WHO Recommendations for
OPV: TRS Nos. 904 and 910 provides more detailed discussions (1).

4.1 Types 1 and 2 Sabin vaccine viruses

The relative sensitivity of the transgenic mouse and monkey tests performed according to WHO
procedures with respect to the presence of mutations in the 5’untranslated region (UTR) in types
1 and 2 appears to be comparable but significantly lower than that in type 3 (2, 3). It is unknown
whether these two models are equally sensitive to other potential neurovirulent mutations. Most
manufacturers use essentially identical seeds of types 1 and 2, in contrast to the situation with
type 3.

4.2 Type 3 Sabin vaccine virus

4.2.1 Molecular biology

Studies of the molecular biology of the Sabin polio vaccine virus strains have suggested that few
mutations are involved in attenuation and that, for the type 3 strain, there may be only two: one
base change in the 5’noncoding region of the genome at base 472 and one coding change at base
2034 that introduces an amino acid change in the virus protein VP3. A third mutation at position
2493 has been described (4). Growth of Sabin 3 virus in cell culture or in vaccine recipients
results in rapid accumulation of U instead of C at nucleotide 2493 (changing Thr to Ile at amino
acid 6 of capsid protein VP1), and all Sabin 3 OPV batches contain variable amounts of these
mutants. This mutation does not affect neurovirulence as determined in the monkey test but there
is evidence that it influences the results obtained in the transgenic mouse test, as described in the
WHO operating procedures (5). Variations in the virulence of vaccine batches measured in
monkeys correlate well with variations in the base in the 5’-UTR as measured by MAPREC.
Changes in the amino acid in VP3, or changes at other positions that suppress its effect, are not
thought to be generated in the course of well controlled production runs, although this is possible
in principle.

4.2.2 Current type 3 seed viruses

Seed viruses currently used for global vaccine production contain variable proportions of the
bases found at position 2493 (C or U):

- The original WHO reference material (passage level SO+2) for neurovirulence
testing contained about an equal mixture of both forms (2493 C or U).
- Batches prepared from RSO, the seeds most commonly used in production in
Europe, typically contain about 5% or less of 2493-U (mutant).
- Seed viruses used in production by some manufacturers (a plaque purified from SO)
contain 100% of mutant form (2493-U) (6).
All OPVs currently in use are believed to have an acceptable safety profile.

5. Experience using the MNVT and TgmNVT with type 3 Sabin seeds and vaccines

There is evidence that the transgenic mouse test, as described in the WHO operating procedures for the TgmNVT, is sensitive to the presence of 2493-U, whereas the monkey test is not sensitive to this mutation. Thus, batches produced from RSO seed will pass both the monkey and transgenic mouse tests, whereas batches produced from the alternative seeds that contain 100% 2493-U will pass the monkey test but may fail the transgenic mouse test, although still having an acceptable safety profile in clinical use.

WHO’s current standard operating procedures for the TgmNVT specify the doses and the WHO reference material to be used and include the proportion of mice affected at the two doses of virus given for the test to be valid. The WHO reference material for TgmNVT is the same as that used in the monkey test and has approximately 50% 2493-C, and it was validated primarily against vaccines made from SO or RSO seeds. However, if used to test vaccines derived from 2493-U containing seed, it may fail them even if they contain little 472-C and would pass MNVT. The TgmNVT could be adapted for testing 2493-U containing bulks – e.g. by changing the reference material, the doses and/or the validity criteria. Manufacturers may wish to do this to make it applicable to their product. Any modified test should be validated and approved by the NRA.

References

Appendix 3

Preparation of poliomyelitis vaccines (oral, live, attenuated) using cell banks – example of a flowsheet

* Control cells: 5% of the total or 500 ml of cell suspension, or 100 million cells.

HAEM = test for haemadsorbing viruses.

CL = cell line used for production, but not the same batch of cells used for production of the virus.

SC = when a human diploid cell line is used for production, a simian kidney cell line should be used as the second indicator cell line. When a simian kidney cell line is used for production, a human diploid cell line should be used as the second indicator cell line (I).

HC = human cells.

Note: This example includes all tests, whether obligatory or not. Since the requirements applicable in a particular place are those authorized by the NRA, this flowsheet should not be considered as an integral part of the requirements and has been included solely for guidance. Manufacturing establishments should prepare their own flowsheet in order to clarify the procedures used.
Appendix 4

Cell-culture techniques for determining the virus content of poliomyelitis vaccines (oral, live, attenuated)

This appendix describes a method for the determination of the virus content of live attenuated OPV in cell cultures. It is an example that is provided for guidance only.

The preparation to be assayed and the reference preparation are diluted in an appropriate medium. It is convenient to make tenfold dilution steps of the virus suspensions initially, but for dilutions that are to be inoculated into Hep-2 (Cincinnati) cell cultures the dilutions should be prepared in 1.0 log\textsubscript{10} or smaller steps. A preliminary assay may be required to ensure that, in the test, the dilution range selected encompasses at least three dilutions that will infect between 0% and 100% of the cultures inoculated.

Titrate the vaccine for infectious virus using no fewer than three separate containers of vaccine following the method described below. Titrate one container of an appropriate virus reference preparation in triplicate to validate each assay. The virus titre of the reference preparation is monitored using a control chart, and a titre is established on a historical basis by each laboratory.

If the vaccine contains more than one poliovirus type, titration of the individual serotypes is undertaken separately using mixtures of appropriate type-specific antisera (or preferably a monoclonal antibody) to neutralize each of the other types present.

For titration of individual serotypes, inoculate a suitable number of wells (ideally 8–10) in a flat-bottomed microtitre plate with equal volumes of the selected dilutions of virus and the appropriate antisera mixture. Total virus content is determined, without any prior incubation, by directly diluting the vaccine in the assay medium. The assay is then incubated for 1–3 hours at 34–36 °C, followed by the addition of an appropriate volume of a suitable cell. The plates are further incubated at 34–36 °C and examined between day 5 and day 9 for the presence of viral cytopathic effect.

The cytopathic effect can be observed by direct reading or after an appropriate staining (vital or fixed staining). The individual virus concentration for each polio serotype and reference preparation is then calculated using an appropriate method.

The assay is considered valid if:

- the estimated virus concentration for the reference preparation is ± 0.5 log\textsubscript{10} CCID\textsubscript{50} of the established value for this preparation;
- the confidence interval (\(P = 0.95\)) of the estimated virus concentration of the three replicates of the reference preparation is not greater than ± 0.3 log\textsubscript{10} CCID\textsubscript{50}.
The assay is repeated and results are averaged if:

- the confidence interval ($P = 0.95$) of the combined virus concentration of the vaccine is greater than ±0.3 log_{10} CCID_{50}.

The assay should be validated for nOPV.
Appendix 5

Model summary protocol for the manufacturing and control of poliomyelitis vaccines (oral, live, attenuated)

The following protocol is intended for guidance and indicates the minimum information that should be provided by the manufacturer to the NRA or NCL. Information and tests may be added or omitted as necessary with the approval of the NRA or NCL. In case the testing method is different from the one listed in this model protocol, it should be approved by the NRA. For example, if molecular methods (such as NAT, HTS) are used for the testing of adventitious agents or mycoplasma, their key parameters and information should be identified and provided, with the minimum of testing method, date of testing, specification and result.

It is possible that a protocol for a specific product may differ in detail from the model provided. The essential point is that all relevant details demonstrating compliance with the licence and with the relevant WHO recommendations on a particular product should be provided in the protocol submitted.

The section concerning the final product must be accompanied by a sample of the label and a copy of the leaflet (package insert) that accompanies the vaccine container. If the protocol is being submitted in support of a request to permit importation, it should also be accompanied by a lot release certificate from the NRA or NCL of the country in which the vaccine was produced and/or released stating that the product meets the national requirements as well as Part A of these WHO Recommendations.

Summary information on finished product (final vaccine lot)

International name: _________________________________________
Trade name: _____________________________________________
Product licence (marketing authorization) number: ____________
Country: _________________________________________________
Name and address of manufacturer: ___________________________
Name and address of licence holder, if different:
Virus strain: _____________________________________________
Origin and short history: _________________________________
Finished product (final lot):
Batch number: _____________________________
Final bulk: _____________________________________________
Type of container: ________________________________
Number of doses per container: _________________________
Number of filled containers in this final lot: ________________
Bulk numbers of monovalent bulk
   Type 1  Type 2  Type 3
suspensions blended in monovalent/bivalent/trivalent vaccine: ________________________________

Site of manufacture of each monovalent bulk: _______________________________________
Date of manufacture of each monovalent bulk: _______________________________________
Date of manufacture of final bulk (blending): _______________________________________
Date of manufacture (filling) of finished product: _______________________________________
Date on which last determination of virus titre was started, or date of start of period of validity: _______________________________________
Shelf-life approved (months): _______________________________________
Expiry date: _______________________________________
Storage conditions: _______________________________________
Volume of human dose (in drops and/or ml): _______________________________________
Virus titre per single human dose:
  Type 1: _______________________________________
  Type 2: _______________________________________
  Type 3: _______________________________________
Nature and concentration of stabilizer: _______________________________________
Nature of any antibiotics present in vaccine and amount per human dose: _______________________________________
Release date: _______________________________________

Summary of source materials
The information requested below is to be presented on each submission. Full details on master and working seed lots should be provided upon first submission only and whenever a change has been introduced.
The following sections are intended for recording the results of the tests performed during the production of the vaccine, so that the complete document will provide evidence of consistency of production. If any test has to be repeated, this must be indicated. Any abnormal result must be recorded on a separate sheet. If any cell lot or virus harvest intended for production is rejected during the control testing, this should also be recorded either in the following sections or on a separate sheet.

Control of source materials (section A.3)

Cell banks (every submission)
Information on cell banking system:
Name and identification of substrate: _______________________________________
Origin and short history: _______________________________________
Authority that approved the cell bank: _______________________________________

| Master cell bank (MCB) and working cell bank (WCB) lot numbers and date of preparation: | ____________________________________________ |
| Date the MCB and WCB were established: | ____________________________________________ |
| Date of approval by national regulatory authority: | ____________________________________________ |
| Total number of ampoules stored: | ____________________________________________ |
| Passage level (or number of population doublings) of cell bank: | ____________________________________________ |
| Maximum passage approved: | ____________________________________________ |
| Storage conditions: | ____________________________________________ |
| Method of preparation of cell bank in terms of number of freezes and efforts made to ensure that a homogeneous population is dispersed into the ampoules: | ____________________________________________ |

**Tests on MCB and WCB, first submission only**

| Percentage of total cell-bank ampoules tested: | ____________________________________________ |

**Identification test:**

| Method: | ____________________________________________ |
| Specification: | ____________________________________________ |
| Date of test: | ____________________________________________ |
| Result: | ____________________________________________ |
| Growth characteristics: | ____________________________________________ |
| Morphological characteristics: | ____________________________________________ |
| Immunological marker: | ____________________________________________ |
| Cytogenetic data: | ____________________________________________ |
| Biochemical data: | ____________________________________________ |
| Results of other identity tests: | ____________________________________________ |

**Tests for adventitious agents**

| Method used: | ____________________________________________ |
| Number of vials tested: | ____________________________________________ |
Tests for bacteria, fungi and mycoplasma

Tests for bacteria and fungi

Method used:

Volume of inoculum per vial:

Volume of medium per vial:

Observation period (specification):

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Media used</th>
<th>Inoculum</th>
<th>Date of start of test</th>
<th>Date of end of test</th>
<th>Results</th>
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<td>20–25 °C</td>
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</tr>
<tr>
<td>30–36 °C</td>
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<tr>
<td>Negative control:</td>
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Test for mycoplasma

Method used:

Volume tested:

Media used:

Temperature of incubation:

Observation period (specification):

Positive controls (list of species used and results):

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<th>Results</th>
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<tr>
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<td>14</td>
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<tr>
<td>21</td>
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</tbody>
</table>

Indicator cell-culture method (if applicable)
Cell substrate used: _______________________________________

Inoculum: _____________________________________________

Date of test: ___________________________________________

Passage number: _________________________________________

Negative control: _______________________________________

Positive controls: _________________________________________

Date of staining: _________________________________________

Results: ________________________________________________

Results of tests for tumorigenicity (if applicable): __________

**Virus seed A.3.2 (Every submission)**

Vaccine virus strain(s) and serotype(s): ___________________________

Substrates used for preparing seed lots: ___________________________

Origin and short history: _______________________________________

Authority data approved virus strains: ___________________________

Date of approval: ___________________________________________

**Information and seed lot preparation, every submission (section A.3.2.1)**

Virus master seed (VMS), virus sub-master seed, and virus working seed (VWS)

Source of VMS: _____________________________________________

VMS and VWS lot number: ___________________________________

Name and address of manufacturer: _____________________________

VWS passage level from VMS: _________________________________

Dates of inoculation: _______________________________________

Dates of harvest: ___________________________________________

Numbers of containers: _____________________________________

Conditions of storage: _______________________________________

Dates of preparation: _______________________________________

Maximum passages levels authorized: ___________________________

**Tests on virus master seed (VMS), virus sub-master seed, and virus working seed (VWS), first submission only**

Test for adventitious agents
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<th>Date of End of Test</th>
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<td>Date(s) of satisfactory test(s) for freedom</td>
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<td>from adventitious agent:</td>
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<tr>
<td>Volume of virus seed samples for</td>
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<tr>
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<tr>
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<tr>
<td>Method used:</td>
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<td><strong>In vitro tests for molecular characteristics</strong></td>
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<tr>
<td><strong>MAPREC (for Sabin OPV)</strong></td>
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<td>Result of test of consistency of production:</td>
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<td>Result of test of comparison with the International Standard:</td>
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<td>Result of test of consistency of production:</td>
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Result of test of comparison with the International Standard: __________________________________________
Type 3
Ratio of %472C of bulk sample to the International Standard or level of mutations: __________________________________________
Result of test of consistency of production: __________________________________________
Result of test of comparison with the International Standard: __________________________________________

**HTS (for virus seed, if applicable)**

<table>
<thead>
<tr>
<th>Type1</th>
<th>Type2</th>
<th>Type3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specification:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Date of test:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Result:</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**In vivo tests for neurovirulence**

**Neurovirulence test in monkeys:**
Result of blood serum test in monkeys prior to inoculation: __________________________________________
Number and species of monkeys inoculated: __________________________________________
Quantity (CCID50) inoculated in each test monkey: __________________________________________
Number of “valid” monkeys inoculated with test sample: __________________________________________
Number of positive monkeys observed inoculated with test sample or with reference: __________________________________________
Reference preparation: __________________________________________
Number of "valid" monkeys inoculated with reference: __________________________________________
Number of positive monkeys observed: __________________________________________
Mean Lesion Score of test sample: __________________________________________
Mean Lesion Score of reference: __________________________________________
(see also attached forms giving details of histological observations and assessment)
C1 constant value: __________________________________________

**Neurovirulence test in transgenic mice**
Strain of mice inoculated: __________________________________________
For each dose of the seed sample: __________________________________________
Number of mice inoculated: __________________________________________
Number of mice excluded from evaluation: _______________________________________

Number of mice paralysed: _______________________________________

Results of validity tests for each dose of the reference virus: _______________________________________
Number of mice inoculated: _______________________________________
Number of mice excluded from evaluation: _______________________________________
Number of mice paralysed: _______________________________________

Virus assay results for each dose inoculated (residual inoculum): _______________________________________
Paralysis rates for test vaccine at each dose: _______________________________________
Paralysis rates for reference virus at each dose: _______________________________________
Results: _______________________________________

Log odds ratio: _______________________________________
L1 and L2 values: _______________________________________
Pass/fail decision: _______________________________________

**Freedom from bacteria, fungi and mycoplasmas**

**Tests for bacteria and fungi**
Method used: _______________________________________
Number of vials tested: _______________________________________
Volume of inoculum per vial: _______________________________________
Volume of medium per vial: _______________________________________

Observation period (specification)_____________________________________

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Media used</th>
<th>Inoculum</th>
<th>Date of start of test</th>
<th>Date of end of test</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>20–25 °C</td>
<td>_______</td>
<td>_______</td>
<td>_______</td>
<td>_______</td>
<td>_______</td>
</tr>
<tr>
<td>30–36 °C</td>
<td>_______</td>
<td>_______</td>
<td>_______</td>
<td>_______</td>
<td>_______</td>
</tr>
<tr>
<td>Negative control</td>
<td>_______</td>
<td>_______</td>
<td>_______</td>
<td>_______</td>
<td>_______</td>
</tr>
</tbody>
</table>

**Test for mycoplasma**
Method used: _______________________________________
Volume tested: _______________________________________
Media used: 

Temperature of incubation: 

Observation period (specification): 

Positive controls (list of species used and results):

<table>
<thead>
<tr>
<th>Subcultures at day 3</th>
<th>Date of start of test</th>
<th>Date of end of test</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subcultures at day 7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subcultures at day 14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subcultures at day 21</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Indicator cell-culture method (if applicable)*

Cell substrate used: 

Inoculum: 

Date of test: 

Passage number: 

Negative control: 

Positive controls: 

Date of staining: 

Results: 

**Virus titration**

Date of test: 

Reference batch number: 

Date of test: 

Result: 

**Genotype characterization**

Method used: 

Date of test: 

Result: 

**Test for mycobacteria**

Method used: 

Date of start of test: 

Date of end of test: 
Result: ____________________________________________

Control of vaccine production (section A.4)

Control of production cell cultures
Lot number of MCB: ____________________________________
Lot number of WCB: ____________________________________
Date of thawing of ampoule of WCB: ______________________
Passage number of production cells: ______________________
Date of preparation of control cell cultures: ______________
Results of microscopic observation: ______________________

Tests on control cell cultures
Ratio of control to production cell cultures: ________________
Incubation conditions: __________________________________
Period of observation of cultures: _________________________
Dates observation started/ended: __________________________
Ratio or proportion of cultures discarded for nonspecific reasons: ________________
Results of observation: _________________________________
Date of supernatant fluid collected: ______________________

Tests for haemadsorbing viruses
Quantity of cell tested: _________________________________
Method used: _______________________________________
Date of start of test: _________________________________
Date of end of test: _________________________________
Results: ____________________________________________

Tests for adventitious agents in cell supernatant fluids
Method used: _______________________________________
Date of start of test: _________________________________
Date of end of test: ______________________________________

Result: ______________________________________

**Identity test**

Method used: ______________________________________

Date of start of test: ______________________________________

Date of end of test: ______________________________________

Result: ______________________________________

**Control of single harvests (section A.4.3)**

Lot number(s): ______________________________________

Date of inoculation: ______________________________________

Temperature of incubation: ______________________________________

Date of harvest: ______________________________________

Volume harvested: ______________________________________

Storage time and approved storage period: ______________________________________

Date of sampling: ______________________________________

**Identity test**

Method used: ______________________________________

Date of start of test: ______________________________________

Date of end of test: ______________________________________

Result: ______________________________________

**Virus titration**

Method used: ______________________________________

Reference batch number: ______________________________________

Date of start of test: ______________________________________

Date of end of test: ______________________________________

Result: ______________________________________

Result of reference: ______________________________________
Tests of neutralized single harvests for adventitious agents

Method used: ____________________________________________

Date of start of test: ______________________________________

Date of end of test: _________________________________________

Result: __________________________________________________

Freedom from bacteria, fungi and mycoplasmas

Tests for bacteria and fungi

Method used: ______________________________________________

Number of vials tested: ______________________________________

Volume of inoculum per vial: _________________________________

Volume of medium per vial: _________________________________

Observation period (specification): ________________________________

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Media used</th>
<th>Inoculum</th>
<th>Date of start of test</th>
<th>Date of end of test</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>20–25 °C</td>
<td>_____</td>
<td>_____</td>
<td></td>
<td></td>
<td>_____</td>
</tr>
<tr>
<td>30–36 °C</td>
<td>_____</td>
<td>_____</td>
<td></td>
<td></td>
<td>_____</td>
</tr>
<tr>
<td>Negative control</td>
<td>_____</td>
<td>_____</td>
<td></td>
<td></td>
<td>_____</td>
</tr>
</tbody>
</table>

Test for mycoplasma

Method used: ______________________________________________

Volume tested: ______________________________________________

Media used: ________________________________________________

Temperature of incubation: __________________________________

Observation period (specification): ____________________________

Positive controls (list of species used and results): ______________

<table>
<thead>
<tr>
<th>Sub cultures at 3rd day</th>
<th>Date of start of test</th>
<th>Date of end of test</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sub cultures at 7th day</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sub cultures at 14th day</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sub cultures at 21th day</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Indicator cell-culture method (if applicable)**

Cell substrate used: ________________________________________

Inoculum: _________________________________________________

Date of test: _______________________________________________

Passage number: ___________________________________________

Negative control: ___________________________________________

Positive controls: ___________________________________________

Date of staining: ___________________________________________

Results: ___________________________________________________

**Test for mycobacteria**

Method used: ______________________________________________

Date of start of test: _________________________________________

Date of end of test: _________________________________________

Result: ___________________________________________________

**Tests for molecular consistency**

**MAPREC (for Sabin OPV, if applicable)**

Date of test: ______________________________________________

Type 1
Ratio of % of the sum of both mutations 480-A, 525-C of bulk sample to the International Standard or level of mutations:
Result of test of consistency of production:
Result of test of comparison with the International Standard:

Type 2
Ratio of % 481-G of bulk sample to the International Standard or level of mutations:
Result of test of consistency of production:
Result of test of comparison with the International Standard:

Type 3

Ratio of % 472C of bulk sample to the International Standard or level of mutations: ____________________________
Result of test of consistency of production: ____________________________
Result of test of comparison with the International Standard: Type1 Type2 Type3

HTS (if applicable)
Specification: ____________________________
Date of test: ____________________________
Result: ____________________________

Control of monovalent bulk (section A.4.4)
Lot number: ____________________________
Date of filtration of bulk: ____________________________
Porosity of filters used: ____________________________
Date of sampling: ____________________________

Identity test
Method used: ____________________________
Date of start of test: ____________________________
Date of end of test: ____________________________
Results: ____________________________
Lot number of reference reagents: ____________________________

Virus titration
Date of test: ____________________________
Reference batch number: ____________________________
Result: ____________________________

Tests for bacteria and fungi
Method used ____________________________
Number of vials tested: ____________________________
Volume of inoculum per vial: ____________________________
Volume of medium per vial: ____________________________
Observation period (specification): ____________________________________________

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Media used</th>
<th>Inoculum</th>
<th>Date of start of test</th>
<th>Date of end of test</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>20–25 °C</td>
<td>_____</td>
<td>_____</td>
<td>_____</td>
<td>_____</td>
<td>_____</td>
</tr>
<tr>
<td>30–36 °C</td>
<td>_____</td>
<td>_____</td>
<td>_____</td>
<td>_____</td>
<td>_____</td>
</tr>
</tbody>
</table>

Negative control: _____ _____ _____ _____ _____

**Test for mycobacteria**

Method used: __________________________________________

Date of start of test: __________________________________

Date of end of test: ____________________________________

Result: ______________________________________________

**Tests for consistency of virus characteristics**

**MAPREC (for Sabin OPV)**

Date of test:

Type 1
Ratio of % of the sum of both mutations 480-A, 525-C of bulk sample to the International Standard or level of mutations:
Result of test of consistency of production:
Result of test of comparison with the International Standard:

Type 2
Ratio of % 481-G of bulk sample to the International Standard or level of mutations:
Result of test of consistency of production
Result of test of comparison with the International Standard:

Type 3
Ratio of % 472C of bulk sample to the International Standard or level of mutations
Result of test of consistency of production:
Result of test of comparison with the International Standard:
### Neurovirulence tests for Sabin OPV

#### Neurovirulence test in monkeys:
- **Result of blood serum test in monkeys prior to inoculation:**
- **Date of inoculation of monovalent bulk:**
- **Number and species of monkeys inoculated:**
- **Quantity (CCID50) inoculated in each test monkey:**
- **Number of “valid” monkeys inoculated with test sample:**
- **Number of positive monkeys observed inoculated with test sample or with reference:**
- **Reference preparation:**
- **Number of "valid" monkeys inoculated with reference:**
- **Number of positive monkeys observed:**
- **Mean Lesion Score of test sample:**
- **Mean Lesion Score of reference:**
  
  *(see also attached forms giving details of histological observations and assessment)*
- **C1 constant value:**

#### Neurovirulence test in transgenic mice
- **Strain of mice inoculated:**
- **For each dose of the bulk sample:**
- **Number of mice inoculated:**
- **Number of mice excluded from evaluation:**
- **Number of mice paralysed:**
- **Results of validity tests for each dose of the reference virus:**
- **Number of mice inoculated:**

<table>
<thead>
<tr>
<th>HTS (if applicable)</th>
<th>Type1</th>
<th>Type2</th>
<th>Type3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specification:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Date of test:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Result:</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

**Neurovirulence tests for Sabin OPV**
Number of mice excluded from evaluation: ____________________________________________
Number of mice paralysed: __________________________________________________________

Virus assay results for each dose inoculated (residual inoculums): ____________________________
Paralysis rates for test vaccine at each dose: _____________________________________________
Paralysis rates for reference virus at each dose: ___________________________________________
Results: __________________________________________________________________________

Log odds ratio: _____________________________________________________________________

L1 and L2 values: ___________________________________________________________________

Pass/fail decision: ________________________________________________________________

**Final bulk A.4.5**

<table>
<thead>
<tr>
<th>Preparation of bulk (types as appropriate):</th>
<th>Type 1</th>
<th>Type 2</th>
<th>Type 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monovalent bulks in blend:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume in blend:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nature and volume of stabilizer:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nature and volume of diluent:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total volume of blend:</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Storage time and approved storage period: ______________________________________________

**Tests for bacteria and fungi**

Method used: ______________________________________________________________________

Number of vials tested: ______________________________________________________________

Volume of inoculum per vial: _________________________________________________________

Volume of medium per vial: _________________________________________________________

Observation period (specification): __________________________________________________

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Media used</th>
<th>Inoculum</th>
<th>Date of start of test</th>
<th>Date of end of test</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>20–25 °C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30–36 °C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Filling and containers (section A.5)

Total volume for final filling: _______________________________________

Date of filling: ____________________________________

Number of vials after inspection: ____________________________________

Number of vials filled: ____________________________________

Control tests on final lot A.6
Inspection of final containers
Appearance: ____________________________________

Date of test: ____________________________________

Results: ____________________________________

Extractable volume
Extractable volume (ml): ____________________________________

The number of drops, using the approved dropper, in a minimum of five individual final containers:

pH
Date of test: ____________________________________

Result: ____________________________________

Identity test
Method used: ____________________________________

Date of start of test: ____________________________________

Date of end of test: ____________________________________

Results: ____________________________________

Lot number of reference reagents ____________________________________

Tests for bacteria and fungi
Method used: ____________________________________

Number of vials tested: ____________________________________

Volume of inoculum per vial: ____________________________________

Volume of medium per vial: ____________________________________

Observation period (specification): ____________________________________

Incubation Media used Inoculum Date of start of test Date of end of test Results
### Virus titration

**Date of test:**

**Reference batch number:**

**Titre of individual virus types:**

**Batch numbers of antiserum used in test:**

**Date of test:**

<table>
<thead>
<tr>
<th>Results</th>
<th>Vaccine</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1:</td>
<td>_______</td>
<td>_________</td>
</tr>
<tr>
<td>Type 2:</td>
<td>_______</td>
<td>_________</td>
</tr>
<tr>
<td>Type 3:</td>
<td>_______</td>
<td>_________</td>
</tr>
</tbody>
</table>

### Thermal stability

**Date of test:**

**Batch numbers of antiserum used in test**

**Results:**

<table>
<thead>
<tr>
<th>Results</th>
<th>Vaccine at 37 °C</th>
<th>Vaccine</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total virus:</td>
<td>_______</td>
<td>_______</td>
<td>_________</td>
</tr>
</tbody>
</table>

### Residual antibiotics (if applicable)

**Date of start of test:**

**Date of end of test:**

**Results:**

### Level of stabilizer (if applicable)

**Date of start of test:**

**Date of end of test:**

**Results:**

**Additional information for production in monkey kidney-cell cultures**

**Production in monkey kidney-cell cultures**

**Control of vaccine production**
Control of monkeys

Monkey species used for production: ________________________________
Quarantine batch number: ________________________________________
Percentage of monkeys surviving quarantine period: ________________
Nature and concentration of antibiotics used in the production cell culture maintenance medium:

Tests for antibodies to simian immunodeficiency virus, SV40, foamy viruses and B virus

Methods used: ________________________________________________
Date of start of test: __________________________________________
Date of end of test: ___________________________________________
Results: ______________________________________________________

Production details

Production monkey number: _________________________________
Date of trypsinizing: _________________________________________
Number of cultures prepared: _________________________________

Cell cultures for vaccine production

Virus seed lot number: ________________________________________
Virus titre/cell ratio: _________________________________________
Number of cultures inoculated: _________________________________
Date of inoculation: _________________________________________
Date of harvest: _____________________________________________
Temperature of incubation: _________________________________
Period of incubation: _______________________________________
Number of cultures harvested: _________________________________

Tests on pooled supernatant fluids

Date of sampling from production cell cultures: ________________
Tests for adventitious agents: _________________________________
Volume tested/cell culture type: 

Observation period: 

Date of completion of tests: 

Results: 

Date of sampling from cell cultures inoculated with the pooled fluid 

Tests for adventitious agents: 

Volume tested/cell culture type: 

Date of completion of tests: 

Results: 

Tests in rabbit kidney-cell cultures 

Volume tested: 

Date of completion of tests: 

Results: 

Control of cell cultures 

Ratio of control to production cell cultures or control cell cultures as a proportion of production cell cultures: 

Period of observation of cultures: 

Ratio or proportion of cultures discarded for nonspecific reasons: 

Results: 

Tests for haemadsorbing viruses 

Methods: 

Results: 

Tests for other adventitious agents 

Methods: 

Results: 

Control of single harvests 

Volume harvested:
Date of sampling:  
Tests for bacteria, fungi, and mycoplasmas:  
Results:  

Tests on neutralized single harvests in monkey kidney-cell and human cell cultures  
Batch number of antiserum used:  
Volume tested:  
Date of starting primary cell culture tests:  
Period of observation:  
Date of sampling cell culture fluids:  
Period of observation:  
Date of completion of tests:  
Results:  

Control of monovalent bulk  

Tests in rabbits  
Number and weight of animals:  
Date of inoculation:  
Results of injection:  
Quantity injected:  
Results (survival numbers, etc.):  
Date of filtration of bulk:  
Porosity of filters used:  
Date of sampling:  

Tests for retroviruses  
Methods:  
Date:  
Results:  

1
2
Certification by the manufacturer

Name of head of production and/or quality control (typed) _____________________________

Certification by the person from the control laboratory of the manufacturing company taking overall responsibility for the production and quality control of the vaccine:

I certify that lot no. ____________ of poliomyelitis vaccine (oral), whose number appears on the label of the final container, meets all national requirements and/or satisfies Part A of the WHO Recommendations to assure the quality, safety and efficacy of live attenuated poliomyelitis vaccine (oral).

Signature: __________________________________________________

Name (typed): ______________________________________________

Date: ____

Certification by the NRA/NCL

If the vaccine is to be exported, attach the model NRA/NCL Lot Release Certificate for poliomyelitis vaccine (oral) (as shown in Appendix 6), a label from a final container and an instruction leaflet for users.
Appendix 6

Model certificate for the release of poliomyelitis vaccines (oral, live, attenuated) by NRAs

This certificate is to be provided by the NRA or NCL of the country in which the vaccine has been manufactured, and/or released on request by the manufacturer.

Certificate no. ________________

The following lot(s) of live attenuated poliomyelitis vaccine (oral) produced by ______________________________(1) in _______________(2) whose numbers appear on the labels of the final containers, comply with the relevant specification in the marketing authorization(3) and the provisions for the release of biological products and Part A(4) of WHO’s Recommendations to assure the quality, safety and efficacy of live attenuated poliomyelitis vaccines (oral) (______)(5) and comply with WHO good manufacturing practices: main principles for pharmaceutical products(6), WHO Good manufacturing practices for biological products(7) and WHO Guidelines for independent lot release of vaccines by regulatory authorities(8).

The release decision is based on _____________________________________________(9).

Final lot number _________________________________________

Number of human doses released in this final lot ___________________________

Expiry date _________________________________________

The certificate may also include the following information:
• name and address of manufacturer;
• site(s) of manufacturing;
• trade name and/or common name of product;
• marketing authorization number;
• lot number(s) (including sub-lot numbers and packaging lot numbers if necessary);
• type of container;
• number of doses per container;
• number of containers or lot size;
• date of start of period of validity (for example, manufacturing date) and/or expiry date
• storage conditions;
• signature and function of the authorized person and authorized agent to issue the certificate;
• date of issue of certificate;
• certificate number.

The Director of the NRA/NCL (or other appropriate authority):
Name (typed) _______________________________________________
Signature __________________________________________________
Date ______________________________________________________

Footnotes:
1 Name of manufacturer.
2 Country of origin.
3 If any national requirements are not met, specify which one(s) and indicate why release of the lot(s) has
nevertheless been authorized by the NRA or NCL.
4 With the exception of provisions on distribution and transport, which the NRA or NCL may not be in a position to
assess.
9 Evaluation of the product-specific summary protocol, independent laboratory testing, and/or specific procedures
laid down in defined document, and so on as appropriate.
Appendix 7

Preparation of poliomyelitis vaccines (oral, live, attenuated) using primary monkey kidney cells – example of a flowsheet

Day 0

| 10 ml MK | (+ serum) |
| 10 ml VK | (fluid) MK |
| 10 ml RK | (+ serum) VK |
| 10 ml HC | |

Pooled fluid (medium change)

Day 6 (2-4) (4-7) 14 19 28

CELL CULTURES (from kidneys of one monkey or no more than 10 near-term monkeys)

Control cell cultures (25%, but not more than 2.5%)

4% HAEM

Pooled fluid (from group)

HAEM

Pooled fluid (from group at medium change)

10 ml MK
10 ml VK
10 ml RK

Day 0 4 18 32

Virus Harvest (14 days after harvest) (28 days after harvest)

Production cell cultures (75%)

Neutralized single harvest

5 ml MK (+ serum) (fluid) MK
5 ml VK (+ serum) VK
10 ml HC

HAEM = test for haemadsorbing viruses.

MK = monkey kidney cells from species (but not the same animal) used for production.

VK = kidney cells from vervet monkey or one sensitive to SV40 virus.

RK = rabbit kidney cells.

HC = human cells sensitive to measles.

Note: This example includes all tests, whether obligatory or not. Since the requirements applicable in a particular place are those authorized by the NRA, this flowsheet should not be considered as an integral part of the requirements and has been included solely for guidance. Manufacturing establishments should prepare their own flowsheet in order to clarify the procedures used.
Appendix 8

International reference materials for poliomyelitis vaccines (oral, live, attenuated)

This appendix describes available international reference materials for OPV, which were developed for Sabin OPV and can be accessed through NIBSC\(^1\) and WHO\(^2\) catalogues. International reference materials for nOPV will be needed, particularly WHO International Standards for the three serotype versions of nOPV should be developed which will likely require monovalent and trivalent formulations. Current neurovirulence references used for MNVT and TgmNVT for Sabin OPV are suitable for nOPV products. Similarly, international standards for anti-poliovirus antibodies, S19 hyper-attenuated poliovirus strains and anti-polio monoclonal antibody sera are suitable for nOPV quality control assays. Finally, specific international reference materials for molecular quality control assays based on HTS will be required for Sabin OPV and nOPV products, respectively.

WHO International Standards and International Reference Preparations are available to ensure manufacture and quality control testing of the different versions of Sabin OPV meet appropriate regulatory requirements.

International Standards for the potency testing of tOPV have been available since 1995. More recently, new International Standards have been established for bOPV, mOPV1, mOPV2 and mOPV3, with compositions and potencies similar to vaccines needed for the final phase of the GPEI.

The 1\(^{st}\) International Standard for the potency estimation of trivalent OPV (NIBSC Catalogue number: 85/659) was established by the WHO ECBS in 1995 as a mixture of three commercially produced monovalent bulks - one of each poliovirus (Sabin) types 1, 2 and 3 (1). Following depletion of stocks of this standard, the 2\(^{nd}\) International Standard was established by the WHO ECBS in 2004 (2), calibrated against International Standard 85/659. The composition of the 2\(^{nd}\) International Standard was kept as close as possible to the 1\(^{st}\) International Standard to allow direct comparison of the two preparations e.g. in stability studies. The 2\(^{nd}\) International Standard (02/306) was also prepared by mixing three commercially produced and released monovalent bulks - one of each poliovirus (Sabin) types 1, 2 and 3. The passage level of the virus in the bulks was: Sabin original (SO) + 3 for type 1, SO+3 for type 2 and a re-derived SO (RSO)+3 for type 3. All three bulks used in the production of this standard were produced on primary monkey cells (PMKC). The standard was prepared by blending the three polio serotypes monovalent bulks in MEM with 1% w/v bovine albumin and sodium bicarbonate buffer. The assigned potency for 02/306 was set at: 7.51, 6.51, 6.87 and 7.66 log\(_{10}\) TCID\(_{50}\)/ml for type 1, 2, 3 and total virus content, respectively. The same bulk materials used to produce the 2\(^{nd}\) International Standard 02/306 were used to prepare candidate preparations for bOPV, mOPV1, mOPV2 and mOPV3 in a similar manner, which were established as International Standards by WHO ECBS in 2017 (3). The 1\(^{st}\) International Standard for

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2 https://www.who.int/activities/providing-international-biological-reference-preparations
bOPV 1+3 (16/164) was assigned potencies 7.19, 6.36 and 7.32 log10 TCID50/ml for type 1, 3 and total poliovirus content, respectively. The 1st International Standards for mOPV1 (16/196), mOPV2 (15/296) and mOPV3 (16/202) were assigned potencies 7.19, 6.36 and 7.32 log10 TCID50/ml for type 1, 2 and 3 poliovirus, respectively. Monoclonal antibody sera against type 1, 2 and 3 poliovirus, 02/256 (NIBSC batch number 425), 02/258 (NIBSC batch number 267) and 02/260 (NIBSC batch number 495), respectively, are available and routinely used globally by a number of manufacturers and NRAs for potency assays of bivalent and trivalent OPV.

Additionally, low titer monovalent type 1, 2 and 3 poliovirus WHO reference reagents are available for use in reference laboratories to measure the sensitivity of cell cultures for poliovirus infection.

Low titer monovalent Sabin type 1, 2 and 3 poliovirus reference reagents were prepared using the same bulk materials used to produce the current International Standards for monovalent, bivalent and trivalent OPV with assigned potencies as follows (4):

- Type 1 (01/528): 5.1 log10 CCID50/0.1ml in RD cells and 4.9 log10 CCID50/0.1ml in L20B cells;
- Type 2 (01/530): 5.1 log10 CCID50/0.1ml in RD cells and 4.8 log10 CCID50/0.1ml in L20B cells;
- Type 3 (01/532): 5.3 log10 CCID50/0.1ml in RD cells and 4.9 log10 CCID50/0.1ml in L20B cells.

Following depletion of stocks of these reagents, new virus reference stocks were prepared from the same original material. The new monovalent reference reagents were established with assigned potencies as follows:

- Type 1 (10/164): 5.5 log10 CCID50/0.1ml in RD cells and 5.3 log10 CCID50/0.1ml in L20B cells;
- Type 2 (10/166): 5.1 log10 CCID50/0.1ml in RD cells and 4.8 log10 CCID50/0.1ml in L20B cells;
- Type 3 (10/168): 5.3 log10 CCID50/0.1ml in RD cells and 4.8 log10 CCID50/0.1ml in L20B cells.

An International Standard for anti-poliovirus types 1, 2 and 3 antibodies (human) is also available for the standardization of neutralizing antibody tests for poliovirus.

The 1st International Standards for anti-poliovirus sera types 1, 2 and 3 were established by the WHO ECBS in 1963 from serotype-specific polyclonal antisera produced by hyper-immunisation of rhesus monkeys with live virus suspensions (5). Each of the standards was specific to one serotype only. They were established through a collaborative study (WHO, 1963) and assigned a unitage of 10 IU/vial, for each of the polio serotypes (5). The 2nd International Standard (66/202) was established by the WHO ECBS in 1991 to replace the 1st International Standards. Stocks of the 1st International Standards ran very low around 1989 and a replacement for the 1st International Standards was selected through a collaborative study (6). In contrast to the 1st International Standard, the 2nd International Standard was a single serum that contained activity against each of the three poliovirus serotypes (7). The following unitage was assigned to the 2nd International Standard: 25 IU of anti-poliovirus serum (type 1) human; 50 IU of anti-poliovirus serum (type 2) human;
and 5 IU of anti-poliovirus serum (type 3) human. Following exhaustion of 66/202, the 3rd
International Standard for anti-poliovirus sera (Human) types 1, 2 and 3 (82/585) was
established by the WHO ECBS in 2006 with assigned unitage of 11, 32 and 3 IU per vial
of neutralising antibody to type 1, 2 and 3 poliovirus respectively (8).

International Standards for MAPREC analysis of poliovirus types 1, 2 and 3 (Sabin) and
International Reference Reagents for control of MAPREC assays of poliovirus type 1, 2 and 3
(Sabin) are available (9). Some of these references might be useful for HTS assays (10) or new
reference might be needed for this purpose.

International Standards and Reference Reagents were prepared from commercial
vaccines and viruses generated by cell culture infection. The list of MAPREC references
currently available is as follows (11-13):
− 00/410: MAPREC assay of poliovirus type 1 (Sabin).100% 480-A, 525-C DNA (1st
   International Reference Preparation).
− 00/416: MAPREC assay of poliovirus type 1 (Sabin) Low Mutant Virus Reference
   (1st International Reference Preparation).
− 00/418: MAPREC assay of poliovirus type 1 (Sabin) (1st International Standard)
− 00/422: MAPREC assay of poliovirus type 1 (Sabin) High Mutant Virus
   Reference(1st International Reference Preparation).
− 97/758: MAPREC analysis of Poliovirus type 2 (Sabin) Synthetic DNA 0.67%481-G
   (1st International Standard)  
− 98/524: MAPREC analysis of Poliovirus type 2 (Sabin) Synthetic DNA, 100%481-G
   (1st International Standard)
− 98/596: MAPREC analysis of Poliovirus type 2 (Sabin) high virus reference 1.21%
   481-G (1st International Reference Preparation)
− 94/790: MAPREC analysis of Poliovirus type 3 (Sabin). Synthetic DNA 100% 472-
   C. WHO (1st International Standard)
− 95/542: MAPREC analysis of Poliovirus type 3 (Sabin) Synthetic DNA 0.9% 472-C.
   WHO (1st International Standard)
− 96/572: MAPREC analysis of Poliovirus type 3 (Sabin) Low virus reference 0.7%472-C
   (1st International Reference Preparation)
− 96/578: MAPREC analysis of Poliovirus type 3 (Sabin) High virus reference 1.1%472-C
   (1st International Reference Preparation)
− 97/756: MAPREC analysis of Poliovirus type 2 (Sabin), low virus reference
   0.65%481-G (1st International Reference Preparation)

Reference preparations at the SO+2 passage level, designated WHO/I for type 1 virus, WHO/II
for type 2 virus and WHO/III for type 3 virus, are available upon request through WHO. These
reference preparations are for use in in vivo neurovirulence tests with vaccines. The relevant
reference materials should be included in each test of vaccine (see section A.4.4.7.2). Virus
panels for validation and implementation of the transgenic mouse neurovirulence test, as
specified in the WHO SOP (14), are also available.

New non-pathogenic, hyper-attenuated poliovirus strains, S19, are available for quality control
assays of OPV (15). S19 strains are polioviruses that replicate in tissue culture but are unlikely to
replicate at all in humans should they be exposed even to large amounts and for this reason they can be used outside GAPIII containment requirements.

The strains are genetically stable and include a portfolio of strains containing the capsid proteins (and thus having the antigenic properties) of the Sabin OPV strains or the wild type strains used most commonly in the production of inactivated polio vaccine. In December 2018, the WHO Containment Advisory Group concluded that the S19 strain can be used outside of the containment requirements of GAPIII for neutralization assays (16). Organizations wishing to use S19 poliovirus strains should follow a detailed validation process to ensure the genetic properties of S19 strains are maintained and can be used to replace current original poliovirus strains. There is a seed-lot system to produce banks of highly characterized S19 strains resembling vaccine production. NIBSC suggests that S19 strains should be tested on a seed lot basis to minimize the risks of reversion and will work with any suitable facility to help generate and validate further banks.

The reference materials listed above are available from the National Institute for Biological Standards and Control, Potters Bar, United Kingdom¹.

References


¹ National Institute for Biological Standards and Control, Potters Bar, United Kingdom: https://www.nibsc.org/


