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

Replacement of: TRS 904, Annex 1 and Addendum TRS 910, Annex 1

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Adopted by the 63rd meeting of the WHO Expert Committee on Biological Standardization, 15 to 19 October 2012. A definitive version of this document, which will differ from this version in editorial but not scientific details, will be published in the WHO Technical Report Series.
Recommendations published by the WHO are intended to be scientific and advisory in nature. Each of the following sections constitutes guidance for national regulatory authorities (NRAs) and for manufacturers of biological products. If a NRA so desires, these 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 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 for additional guidance intended for manufacturers and NRAs, which may benefit from those details.
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Introduction

Requirements for oral poliomyelitis vaccine (OPV) were first formulated in 1962 (I) 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 PCR and restriction enzyme cleavage (MAPREC) assay as an optional, additional in vitro test of poliovirus type 3, increased levels of laboratory containment of wild polioviruses (9) 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 last revised in full in 1999 (10) when the use of transgenic mice expressing the human poliovirus receptor (TgPVR21 mice) (11) as an alternative to the MNVT for type 3 virus were included in the revision and the MAPREC test was introduced as the in vitro test of preference for the evaluation of filtered bulk suspensions for poliovirus type 3 (12). The rct40 test then became an optional, additional test. 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 (13) to include the neurovirulence test in TgPVR21 mice as an alternative to the MNVT for all three poliovirus serotypes.

Since then there have been advances in scientific knowledge, novel laboratory techniques have become available, and new vaccine formulations such as monovalent/bivalent OPV are being used. In 2008, the WHO Expert Committee on Biological Standardization advised that the recommendations for OPV should be revised. In addition, various tests are now applicable to all three types of polioviruses and their significance needs to be better explained and rationalized. Sections on nonclinical and clinical evaluation for new candidate OPVs are also required. To facilitate this process, WHO convened a working group meeting to initiate the revision of the recommendations for the production and control of OPV, as outlined in Nos. 904 and 910 of the WHO Technical Report Series. Experts from academia, national regulatory authorities (NRAs)/national control laboratories (NCLs) and industry involved in the research, manufacture, authorization and testing/release of OPV from countries around the world met on 20–22 July 2010 to identify and discuss the issues to be considered in the revision of the WHO Technical Reports Nos. 904 and 910 (14).

Major issues addressed during this revision include:

- an update on the origin of different strains for OPV production with inclusion of a new Appendix 1;
- an update of the section on international standards and reference preparations;
- an update of the section on general manufacturing recommendations and control tests;
- an update on neurovirulence tests (MNVT and TgmNVT) and the MAPREC test which is extended to all three types of seeds and bulks; and inclusion of a new
Appendix 2 giving rationales for the choice of monkey or mouse neurovirulence tests;
- consideration of new vaccine formulations (mOPV and bOPV);
- an update on terminology, and the introduction of the “virus sub-master seed lot” which is applicable only to the master seed supplied by WHO;
- inclusion of new sections on nonclinical and clinical evaluation of OPV;
- an update on appendices;
- an update on the standard operating procedures (SOPs) for TgmNVT and MAPREC and new MNVT in light of current developments in techniques.

Additional changes have been made to bring the document into line with other WHO recommendations published since the last revision.

Scope

The scope of the present 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 Sabin poliovirus strains regardless of their history. It does not necessarily apply to other strains should they be developed.

This document should be read in conjunction with the relevant WHO guidelines, such as those on nonclinical (15) and clinical evaluation (16) of vaccines.

General considerations

Poliomyelitis is an acute communicable disease of humans caused by three distinct poliovirus serotypes called types 1, 2 and 3 and distinguished by a neutralization test (17). Poliovirus is a species C human enterovirus of the Picornaviridae family and is composed 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 settings with a higher standard of sanitation. However, in most settings, mixed patterns of transmission are likely to occur. In the pre-vaccine era, roughly one out of 200 susceptible individuals infected by polioviruses developed paralytic poliomyelitis (17).

Progress in polio control (and, since 1988, polio eradication) has been due mainly to widespread use of vaccines. An inactivated poliovirus vaccine (IPV Salk vaccine) was licensed in 1955; live, attenuated OPV (Sabin vaccine) was licensed as monovalent OPV in 1961 and as trivalent OPV (tOPV) in 1963. The Sabin strains of poliovirus used in the production of 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 the latter provided many advantages, including ease of administration, suitability for mass vaccination campaigns, superior induction of 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. By 2010, three of the six WHO regions had been certified as free of wild polio viruses, and wild type 2 has not been detected worldwide since 1999 (17).
In addition to tOPV, which is used in many countries for routine or supplementary vaccination, monovalent OPVs against type 1 (mOPV1) and against type 3 (mOPV3) and bivalent OPV against type 1 and type 3 (bOPV) (17), which are used by the Global Polio Eradication Initiative, have been licensed for use in endemic countries or for outbreak control in situations where one or two types can re-emerge. Monovalent OPV against type 2 has been licensed but is expected to be used primarily for emergency response stockpiles. Recently, the Strategic Advisory Group of Experts (SAGE) on Immunization was asked by WHO to consider the possible replacement of tOPV with bOPV for routine immunization globally.

Following the introduction and widespread use of the first mOPV1 and mOPV3 in supplementary immunization activities in 2005, the polio eradication programme has reported substantial reductions in the respective poliovirus types. The last case in India was in January 2011 and, as polio is now considered to have been eradicated there, India has been removed from the list of endemic countries. However, the co-circulation of wild poliovirus types 1 and 3 in the three remaining polio-endemic countries requires huge quantities of bOPV to be used to supplement tOPV given in routine and mass campaigns. A clinical trial to evaluate the immunogenicity of different OPV formulations (mOPV1, mOPV3 and bOPV) compared to tOPV in an Indian population was conducted by WHO. The seroconversion rates to poliovirus type 1 and type 3 following immunization with bOPV were significantly higher than those induced by tOPV and were not lower than those induced by immunization with either mOPV1 or mOPV3 respectively (18).

Although OPV is a safe vaccine, adverse events may occur on rare occasions (17). Vaccine-associated paralytic poliomyelitis (VAPP) is the most important of these rare adverse events. Cases of VAPP are clinically indistinguishable from poliomyelitis caused by wild poliovirus, but can be distinguished by laboratory analysis. The incidence of VAPP has been estimated at four cases per million birth cohort per year in countries using OPV (19). 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 wild-strain poliovirus (WPV) and can cause polio cases and outbreaks as circulating vaccine-derived poliovirus (cVDPV) (20).

Live vaccines prepared from the Sabin strains of poliomyelitis viruses of types 1, 2 and 3 were introduced for large-scale immunization in 1957. 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 strains prepared by Behringwerke has been published by Cockburn (21). 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.

The original poliovirus seeds produced by Sabin (SO) (22) 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.

The type 3 vaccine was found to be less stable on passage than either type 1 or type 2, which was manifested in a higher number of type 3 vaccine lots failing the monkey neurovirulence test. In order to develop a more stable strain, a new seed was prepared by Pfizer by transfecting susceptible cells with viral RNA extracted from poliovirus at the SO+2 level. One plaque, which was designated 457-III, was identified with particularly favourable properties (23). 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 in the course of 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 sequence of RSO has been shown to be the same as the consensus of SO (24), but it was more homogeneous and contained lower quantities of mutant viruses.

The RSO seed was not used for the production of type 3 vaccine until the 1980s when it became clear that the stocks of material 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 Merieux, Pasteur Merieux Connaught and subsequently Aventis Pasteur) which has recently donated them to WHO.

The virus seeds available from WHO (designated “the WHO master seeds”) are therefore types 1, 2 and 3 at SO+1 level produced by Behringwerke from SO seeds and the type 3 RSO seed donated by Sanofi Pasteur. The seeds are kept at the National Institute for Biological Standards and Control in the United Kingdom, and include a proportion of the stocks of the SO+1 seeds formerly held at Istituto Superiore di Sanità in Italy which kindly transferred them (21, 23).

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 plaque purification (cloning). Sequencing of these seed viruses demonstrated that, while they contained low content of neurovirulent mutants, there were differences between these strains and the consensus sequence of Sabin Original virus (24). However, there are no reports of any differences in clinical safety 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 as compared to an appropriate reference preparation (25). 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 (26, 27). 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;
- in some countries, a reduction in the 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 Japan Poliomyelitis Research Institute and at the Center 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 (28). 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) (10) 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 (13). Laboratories must comply with specifications for containment of the transgenic animals (29). 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. Evidence strongly suggests that mutations in the 5’ noncoding region of the poliovirus genome, especially for the Sabin type 3 strain, are critical in determination of the attenuated phenotype (30). A molecular biological test, MAPREC assay, was developed by researchers at CBER to quantify reversion at the molecular level (31). 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 (32).

In 1991, WHO initiated a series of international collaborative studies to evaluate the MAPREC assay for all three types of poliovirus 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 the 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) (10) 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 for comparable positions in type 1 and type 2 have now been established. While the results do not correlate with neurovirulence in the range studied, they provide a measure of production consistency. The quantity of other mutants (such as 2493-U in Sabin 3 virus) can also be used to identify types of seed virus and to monitor...
consistency of manufacture. After appropriate validation, quantitative profiles of other mutations in stocks of OPV could be used for this purpose.

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, although the vaccines produced from human diploid cells or continuous cell lines have been used to a lesser extent than those produced in cultures of primary monkey kidney cells, experience has indicated that all three cell substrates 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 (33). For such products, the principal requirement is the elimination of potentially contaminating viruses. Recently, additional data on the uptake of DNA via the oral route have been published (34). 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 the NRA (35) and, where possible, data should be accumulated on the levels of rcDNA in OPV produced in Vero cells.

At the time of preparation of this document, there was increasing interest in developing, through molecular manipulation, alternative strains for use in OPV production. The poliovirus-specific quality evaluation of such strains – e.g. neurovirulence testing, MAPREC etc – as described in these recommendations and associated SOPs, may not be appropriate. The tests on such vaccines, which are likely to include extensive preclinical and clinical studies to demonstrate attenuation, genetic stability, safety and transmissibility of the proposed strains, will have to be considered separately on a case-by-case basis and may differ fundamentally from those described in this document.

Part A. Manufacturing recommendations

A.1 Definitions

A.1.1 International name and proper name
The international name should be live attenuated poliomyelitis vaccine (oral) with additions to indicate the virus serotype(s) of the vaccine. The proper name should be equivalent to 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 specifications formulated below.

A.1.2 Descriptive definition
Live attenuated poliomyelitis vaccine (oral) is a preparation of live attenuated poliovirus types 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, prepared in a form suitable for oral administration and satisfying all the recommendations formulated in this document.

A.1.3 **International reference materials**

A trivalent virus mixture is available as an International Reference Reagent for Live Attenuated Poliovirus (Sabin) Types 1, 2 and 3 for determination of virus titre.

Three monotypic virus suspensions of types 1, 2 and 3 have been established as WHO reference reagents for use in reference laboratories to measure the sensitivity of cell cultures for poliovirus infection.

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.

International Standards for anti-poliovirus types 1, 2 and 3 antibodies (human) are available for standardization of neutralizing antibody tests for poliovirus.

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

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 from WHO.

1 These reference preparations are for use in in vivo neurovirulence tests with homotypic vaccines. The relevant reference materials should be included in each test of vaccine (see section A.4.4.7.2).

A.1.4 **Terminology**

The definitions given below apply to the terms as used in these recommendations. They 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.

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1 Contact the Coordinator, Quality, Safety and Standards, World Health Organization, 1211, Geneva 27, Switzerland (http://www.who.int/biologicals/vaccines/en/).
**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 of 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 −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.

**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-derived Sabin Original type 3 virus (23). All subsequent passages are designated by an additional number – e.g. RSO1 (master seed) is one passage on from RSO. The working seed passage level is therefore RSO2 and the vaccine RSO3.

**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 (22). 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 (only applicable for master seed supplied by WHO):** A quantity of virus suspension produced by a single passage from the virus master seed supplied by WHO 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. The virus sub-master seed lot should be characterized to the extent necessary 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 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 by a method approved by the NRA.

**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 aliquots, 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.
A.2 General manufacturing recommendations

The general manufacturing recommendations contained in *Good manufacturing practices for pharmaceutical products: main principles* (36) and *Good manufacturing practices for biological products* (37) should apply to establishments manufacturing OPV, with the addition of the following:

- The production of OPV should be conducted by staff who should be healthy persons and who should be examined medically at regular intervals. Steps should be taken to ensure that all such 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 freeze-dried BCG vaccine* (38).
- The establishment should be in compliance with the current global recommendations for poliovirus containment.

A.3 Control of source material

General production precautions, as formulated in *Good manufacturing practices for biological products* (37) should apply to the manufacture of OPV, with the addition that, during production, only one type of cell should be introduced or handled in the production area at any one time. Vaccines may be produced in cell lines such as MRC-5 and Vero cells (see section A.3.1) or in primary monkey kidney cells (PMKC) (see Part E).

A.3.1 Cell lines

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* (35). 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 (35).

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 (39) and is available to manufacturers on application to the Coordinator, Quality, Safety and Standards, World Health Organization, Geneva, Switzerland.

A.3.1.2 Identity test

Identity tests on the master (MCB) and working cell banks (WCB) are performed in accordance with WHO’s *Recommendations for the evaluation of animal cell cultures as substrates for the*
The WCB should be identified by means of, inter alia, biochemical tests (e.g. isoenzyme analysis), immunological tests, cytogenetic marker tests and DNA fingerprinting or sequencing. 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 (40) and 5.3 (41) of the General requirements for the sterility of biological substances, and freedom from infectious viruses. Suitable tests for detecting viruses in bovine serum are given in Appendix 1 of 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 (35).

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 phage and endotoxin. Gamma irradiation may be used to inactivate potential contaminant viruses, 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 (42).

Human serum should not be used. If human serum albumin is used at any stage of product manufacture, the NRA should be consulted regarding the requirements, as these may differ from country to country. As a minimum, the serum should meet the Requirements for the collection, processing and quality control of blood, blood components and plasma derivatives (43). 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 (42).

Penicillin and other beta-lactams should not be used at any stage of manufacture because of their nature as highly sensitizing substances.

Other antibiotics may be used at any stage in the manufacture provided that the quantity present in the final lot is acceptable to the NRA.

Nontoxic pH indicators may be added, such as phenol red in 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 free of cultivable bacteria, fungi, mycoplasmas and infectious viruses, as appropriate. The methods used to ensure this should be approved by the NRA.

In some countries, irradiation is used to inactivate potential contaminant viruses. 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 for the biological properties of the reagents to be retained but also high enough to reduce virological risk. Therefore, irradiation cannot be considered a sterilizing process (35).

Recombinant trypsin is available and should be considered; however, it should not be assumed to be free from risk of contamination and should be subject to the usual considerations for any reagent of biological origin (35).

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 (42).

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, which should include information on their origin. Producers of OPV can obtain virus master seeds from WHO. Manufacturers receiving this virus may prepare a sub-master seed by a single passage and then prepare their working seed. However, 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 master seed lot and virus working seed lot used for the production of vaccine batches should be prepared by a single passage from the virus strain and the virus master seed lot respectively, by a method and at a passage level from the original seed virus approved by the NRA. A virus sub-master seed lot may be prepared by a single passage from the WHO master seed, and the characterized virus sub-master seed lot (see Part B) may be used for the preparation of virus working seed lots by a single passage.

Virus master, sub-master and working seed lots should be stored in dedicated temperature-monitored freezers at a temperature that ensures stability on storage e.g. ≤−60 °C. 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 virus master seed is provided by WHO as well characterized seed material. The virus sub-master and working seed lot used for the production of vaccine batches should be shown to be free from detectable extraneous viruses and from detectable SV40 DNA, as determined by a validated nucleic acid amplification test, and should be in conformity with the recommendations set out in Part A, sections A.4.3 (Single harvests) and A.4.4.1, A.4.4.2, A.4.4.3 and A.4.4.4 (Monovalent bulks). The control cell cultures should conform to section A.4.1 (Control of cell cultures).

DNA of SV40 is widely used as molecular biological reagent, and contamination of polymerase chain reaction (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.

A.3.2.4 Tests to monitor virus molecular characteristics
A.3.2.4.1 Tests in vitro
Seed viruses should be tested in MAPREC or temperature sensitivity assays (rct40) (see section A.4.4.7.1). At least three consecutive monovalent bulks prepared from the seed virus should meet the criteria for acceptability given in section A.4.4.7.1, with the agreement of the NRA. Historically, four consecutive monovalent bulks prepared from the seed virus have been tested for monitoring the virus molecular characteristics and production consistency.

A.3.2.4.2 Neurovirulence tests
New virus working seeds should be evaluated for neurovirulence. 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, and transgenic mice, nonhuman primates, or both may be used.

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

The use of the TgmNVT should be approved by the NRA and 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).

Under normal circumstances, a new virus working seed will be prepared using the same production protocol and from the same virus 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.

In case there are any major changes in the production process for a new virus master seed, full characterization in tests in nonhuman primates and transgenic mice 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.

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1 Contact the Coordinator, Quality, Safety and Standards, World Health Organization, 1211, Geneva 27, Switzerland (http://www.who.int/biologicals/vaccines/en/).
Historically, four consecutive monovalent bulks prepared from the seed virus have been tested in monkeys for monitoring the production consistency.

A.3.2.5 Genotype characterization
Advances have been made in the development and application of molecular methods such as deep sequencing. For any new virus working seed, it may be useful for information purposes to analyse the new virus working seed and at least three consecutive monovalent bulks for nucleotide sequence changes from the seed virus (deep genome sequence). If such tests are performed for regulatory purposes, they should be scientifically validated and approved by the NRA.

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

A.4.1.2 Tests for haemadsorbing viruses

At the end of the observation period, 25% of the control cells should be tested for the presence of haemadsorbing viruses using guinea pig red blood cells. If these cells have been stored, the
duration of storage should not have exceeded seven days and the storage temperature should have been in the range 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 30 minutes’ incubation at 2–8 °C and again after a further incubation for 30 minutes at 20–25 °C.

If a test with monkey red cells is performed, readings should also be taken after a final incubation for 30 minutes at 34–37 °C.

A.4.1.3 Tests for other adventitious agents in cell fluids

At the end of the observation period, a sample of the pooled 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 (35).

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 cells 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.

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) (35).
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 are, 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 in a cell culture 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, within the range 33–35 °C, for the relevant incubation periods. The temperature should 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 for each manufacturer and should be approved by the NRA.

The virus suspension should be harvested not later than four days after virus inoculation.

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 as the appropriate poliovirus serotype by immunological assay on cell culture using specific antibodies or by a molecular method which has been validated and approved by the NRA.
Neutralization tests can distinguish the serotypes of poliovirus. Molecular method such as sequencing or deep sequencing, can distinguish Sabin virus from wild-type virus. 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.4.3.2 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 Tests of neutralized single harvests for adventitious agents**

Some selected viruses may be screened by using specific assays such as molecular techniques (e.g. nucleic acid amplification) (35). 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 microarrays of conserved viral sequencing or digital subtraction of expressed sequences; and high throughput sequencing. 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 approval of the NRA (35).

A.4.3.4 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 (41) and 5.3 (41) of the General requirements for the sterility of biological substances, or by a method approved by the NRA.

Nucleic acid amplification techniques (NAT) alone or in combination with cell culture, with an appropriate detection method, may be used as an alternative to one or both of the compendial mycoplasma detection methods following suitable validation and agreement from the NRA (35).

A.4.3.5 Test for mycobacteria
The virus harvest should be shown to be free from mycobacteria by an appropriate method approved by the NRA.

Molecular assays 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 (35).

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

A.4.3.6 Tests for molecular consistency of production
Some manufacturers perform a test for the molecular consistency of production, namely the MAPREC assay (see section A.4.4.7.1.1) on single harvests. If performed, the acceptance and rejection criteria of this test should be updated periodically and approved by the NRA.

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 as the appropriate poliovirus serotype by immunological assay on cell culture using specific antibodies, or by a molecular method which has been validated and approved by the NRA.

Neutralization tests can distinguish the serotypes of poliovirus. Molecular methods, such as sequencing or deep sequencing, can distinguish Sabin virus from wild-type virus.

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.4.4.4 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 TgPVR 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 (40).

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 may be used as an alternative to mycobacteria microbiological culture method tests for the detection of mycobacteria after suitable validation and agreement from NRA (35).

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. The test used should be approved by the NRA. The MAPREC assay provides a sensitive and quantitative measure for consistency purposes. However, other assays are acceptable after validation. The assay used historically is to test the property of reproducing virus at temperatures of 36°C and 40°C in comparison with the seed lot or a reference virus preparation of poliovirus of the same type.

A.4.4.7.1.1 MAPREC
The MAPREC assay is suitable for all three serotypes. 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. 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 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 should be approved by the NRA.

Levels of mutations obtained by manufacturers who have implemented the test 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) (14).

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 (14).

1 Contact the Coordinator, Quality, Safety and Standards, World Health Organization, 1211, Geneva 27, Switzerland (http://www.who.int/biologicals/vaccines/en/).
Non-radioactive methods for MAPREC are available and may be introduced after validation and approval by the NRA.

Alternative molecular biology methods that demonstrate an equivalent or better level of discrimination may be used after validation and approval by the NRA.

A.4.4.7.1.2 Temperature sensitivity

The monovalent bulk may be tested for the property of reproducing at temperatures of 36 °C and 40 °C in comparison with the seed lot or a reference virus preparation for the marker tests, and with appropriate rct/40- and rct40+ strains of poliovirus of the same type. The wild-type viruses, defined as field isolates or reference strains from polioviruses known or believed to have circulated persistently in the community, which are used as rct40+ controls in this test, should be contained within the laboratory at progressively higher levels of containment in accordance with the global action plan and timetable for safe handling of wild polioviruses. The incubation temperatures used in this test should be controlled to within ±0.1 °C.

The monovalent bulk passes the test if, for both the virus in the monovalent bulk and that in the appropriate reference material, the titre determined at 36 °C is at least 5.0 log₁₀ greater than that determined at 40 °C. If the titres obtained for all the reference viruses are not in line with the expected values, the test should be repeated.

An additional specification that the virus titre must not exceed 10 CCID₅₀/ml at the higher temperature may also be applied.

It is desirable that the temperatures used in the test should also include one in the region of 39.0–39.5 °C, at which the titre of the reference material should be reduced by a factor in the range of 3.0–5.0 log₁₀ of its value at 36 °C. In one laboratory, a temperature of 39.2 °C has been found suitable.

It is important to show that the behaviour of the monovalent bulk is comparable to that of the Sabin reference strain over a range of temperatures so that a more accurate comparison can be made.

A.4.4.7.2 Neurovirulence tests

An appropriate in vivo test should be used to evaluate virus seeds and 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.¹

¹ Contact the Coordinator, Quality, Safety and Standards, World Health Organization, 1211, Geneva 27, Switzerland (http://www.who.int/biologicals/vaccines/en/).
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 test, there is a requirement for laboratories to complete a standard implementation process as detailed in the 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 (28). 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.

A.4.5 Final bulk

Different final bulks can be formulated.

Final tOPV bulk, final mOPV1 bulk, mOPV3 bulk and final bOPV bulk (bOPV1+3) can be manufactured using a defined virus concentration of each component.

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 added to 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.

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 (40).

A.5 Filling and containers

The requirements concerning filling and containers given in Good manufacturing practices for biological products (37) should apply to vaccine filled in the final form.

Care should be taken that the material of which the container is made does not adversely affect the virus content of the vaccine under the recommended storage conditions.
A final filtration may be included just before the filling operations.

The manufacturer should provide the NRA with adequate data to prove that the product is stable under appropriate conditions of storage and shipping.

### A.6 Control tests on the final lot

Samples should be taken from each filling lot for the tests described in the following sections. The following tests should be performed on each final lot of vaccine (i.e. 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. The permissible limits for the different parameters listed under this section, unless otherwise specified, 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.

##### A.6.1.1 Appearance

The appearance of the vaccine should be described 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.

#### A.6.3 pH

The pH of the final lot should be tested in a pool of final containers and an appropriate limit set to guarantee virus stability.

#### A.6.4 Identity

Each final lot should be identified by immunological assay on cell culture using specific antibodies or by a molecular method which has been validated and approved by the NRA.

Neutralization tests can distinguish the serotypes of poliovirus. Molecular methods such as sequencing or deep sequencing, can distinguish Sabin virus from wild-type virus.

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 used for this purpose.

#### A.6.5 Sterility tests for bacteria and fungi
Liquid vaccine 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* (40), or by the 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. The poliovirus content of each serotype, and the total virus content, should be determined in an assay, as described in Appendix 4 of these recommendations, using assays that include a reference preparation. 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 NRA should specify the minimum virus titre per human dose.

An internal upper limit may be established by each manufacturer to monitor the consistency of production (e.g. based on mean titre CCID\textsubscript{50} +3 standard deviations). The upper limit should be approved by the NRA.

It is recommended that the estimated mean virus titres for a single human dose of tOPV should be not less than $10^{6.0}$ CCID\textsubscript{50} for type 1, $10^{5.0}$ CCID\textsubscript{50} for type 2, and $10^{5.5}$ CCID\textsubscript{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\textsubscript{10} of the estimated number of infectious units in the vaccine.

In 1986 the WHO Region of the Americas began to use a trivalent formulation with $10^{5.8}$ CCID\textsubscript{50} of poliovirus type 3 (44), following a study in Brazil which demonstrated improved immunogenicity when the amount of type 3 virus in the trivalent vaccine was increased (45). 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\textsubscript{50} per dose for types 1, 2 and 3 respectively, on a global basis (18, 46).

### 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* (47).

Three final containers of the vaccine 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. For trivalent vaccines, the vaccine passes the test when the loss on exposure is not greater than a factor of 0.5 log\textsubscript{10} CCID\textsubscript{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 $-20\,^\circ\mathrm{C}$ or below and six months when stored at $2-8\,^\circ\mathrm{C}$.

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 8 of *Good manufacturing practices for biological products* (37) should apply.

A.8 Retained samples

The requirements given in section 9.5 of *Good manufacturing practices for biological products* (37) should apply.

A.9 Labelling

The requirements given in section 7 of *Good manufacturing practices for biological products* (37) should apply, with the addition of the following.

The label on the container or package 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 section 8 of Good manufacturing practices for biological products (37) should apply. Further guidance is provided in WHO’s Model guidance for the storage and transport of time- and temperature-sensitive pharmaceutical products (48).

A.11 Stability, storage and expiry date

A.11.1 Stability testing

Adequate stability studies form an essential part of vaccine development. Current guidance on the evaluation of vaccine stability is provided in WHO’s Guidelines on stability evaluation of vaccines (47). Stability testing should be performed at different stages of production, namely on single harvests, monovalent bulk, final bulk and final lot. Stability-indicating parameters should be defined or selected appropriately according to the stage of production. A shelf-life should be assigned to all in-process materials during vaccine production – particularly intermediates such as single harvests, monovalent bulk and final bulk.

The stability of the vaccine in its final container and at the recommended storage temperatures 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 give additional information on the overall characteristics of a vaccine.

The formulation of vaccine should be stable throughout its shelf-life. Acceptable limits for stability should be agreed with NRAs. Following licensure, ongoing monitoring of vaccine stability is recommended to support shelf-life specifications and to refine the stability profile (47). Data should be provided to the NRA in accordance with local regulatory requirements.

Where 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 approved by the NRA.

The final stability testing programme should be approved by the NRA and should include an agreed set of stability-indicating parameters, procedures for the ongoing collection and sharing of stability data, and criteria for the rejection of vaccine(s).

A.11.2 Storage conditions

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

The manufacturer should indicate conditions of storage and shipping that will ensure that the vaccine conforms to the requirements of potency until the expiry date stated on the label. These must be approved by the NRA.
Although the recommended storage temperature is at $-20\, ^\circ C$, vaccine may be stored at 2–8°C for six months. During shipment or in the field, the vaccine may be thawed and refrozen.

Manufacturers should demonstrate that multiple freeze–thaw cycles do not adversely affect the quality of the product. The number of freeze–thaw cycles should be approved by the NRA.

The total storage period at 2–8 °C should not exceed six months. Stability data should be generated for each formulation of OPV to support these excursions in the storage conditions at 2–8 °C following thawing, and this should be approved by the NRA.

A.11.3 Expiry date
The expiry date should be defined on the basis of the shelf-life and should be supported by the stability studies with the approval of the NRA. The expiry date should relate to the date of filling or the date of the first valid titration for virus content after filling, performed in an assay of virus concentration as described in Appendix 4 (i.e. the date of the potency test).

The label should specify only one storage temperature and expiry date.
Part B. Nonclinical evaluation of live attenuated poliomyelitis vaccines (oral)

The nonclinical evaluation of candidate live attenuated poliomyelitis vaccines (oral) should be based on WHO guidelines on nonclinical evaluation of vaccines (15). The following specific issues should be considered in addition to the tests described in sections A.3.2.3 and A.3.2.4 in the context of a change in virus seed or manufacturing process for OPV.

B.1 Characterization of a new virus sub-master seed from the WHO master seed

In the event that a new virus sub-master seed is prepared by a single passage from 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. Massively parallel sequencing may also be undertaken to determine the distribution of mutants. These approaches have not yet been formally validated, other than the MAPREC tests for base positions in the 5’ noncoding region, as described in section A.4.4.7.1.1. A new sub-master seed should be tested for neurovirulence in the MNVT or the TgmNVT. Summaries of the MNVT and TgmNVT are given in Appendix 2 along with considerations on the choice of assay.

B.2 Characterization of virus working seeds from an established master seed where passage level between master and working seed is increased

The acceptable passage level of live polio vaccines relative to the original seeds is rigidly specified as there is evidence for some seeds that increases in virulence have occurred with increasing passage. However, due to the limited stocks of master seeds available, it may be necessary in the future for some manufacturers to prepare working seed lots by expanding current seed lots with an additional passage. The new virus working seed lots will require careful comparative studies with the previously approved working seed lot and will need to meet the criteria outlined in sections A.3.2.3 and A.3.2.4. At least three monovalent bulks produced from the new virus working seed lot should also be tested and shown to meet the requirements of section A.4.4.7.

B.3 Characterization following changes in the manufacturing process

If the OPV manufacturing process is new or major changes are implemented in vaccine production – such as changing from primary monkey cells to cell lines – extensive assessment should be conducted to ensure that the mutational composition is not significantly altered by the new manufacturing process. This evaluation may include nucleotide sequencing and studies of mutant accumulation during passage in production cultures by using MAPREC and other molecular methods such as massively parallel sequencing. The new virus working seed lots will meet the criteria outlined in sections A.3.2.3 and A.3.2.4. At least three monovalent bulks
produced from it also need to be tested and shown to meet the requirements of section A.4.4.7. In addition, based on the results of the genetic characterization and the animal neurovirulence tests, clinical studies may be required (see Part C).
Part C. Clinical evaluation of live attenuated poliomyelitis vaccines (oral)

Clinical trials should adhere to the principles described in WHO’s Guidelines for good clinical practice (GCP) for trials on pharmaceutical products (49) and Guidelines on clinical evaluation of vaccines: regulatory expectations (16). 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:

- new formulations based on licensed OPV that is based on 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 by using a new virus working seed lot provided that the passage level is not more than one from the master seed lot, the working seed has been characterized, and consistency of the manufacturing process has been demonstrated (see sections A.3.2.3 and A.3.2.4). Generation of a new sub-master seed will require extensive characterization but not clinical trials (see Part B).

Vaccine formulations containing one or two poliovirus serotypes have been licensed on the basis of 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 (18, 50). 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 “non-inferior” to mOPV1 and mOPV3 individually, and “superior” to tOPV. Therefore, based on these results, the Advisory Committee on Poliomyelitis Eradication (ACPE) recommended that bOPV should be used to complement tOPV in routine immunization and to complement tOPV and mOPVs in supplementary immunization activities.

C.1 General considerations

The poliomyelitis eradication initiative which followed World Health Assembly resolution WHA41.28 of 1988 has led to a dramatic decrease in poliomyelitis cases globally (17). Therefore, efficacy studies for poliovirus vaccines are not feasible, and clinical evaluation and seroprevalence studies should be based on comparative assessment of the safety and immunogenicity of new 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 (17). The approval of a new candidate OPV should be based on a clear demonstration of non-inferiority compared to currently licensed OPV. 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.

C.2 Safety and immunogenicity studies

C.2.1 Assessment of the immune response

A serum neutralizing antibody titre of 1/4–1/8 is considered to be a marker of protection against poliovirus (51). The demonstration of an immune response to OPV vaccination should be based on the measurement of neutralizing antibody titres pre- and post-vaccination. Geometric mean titres (GMTs), seroconversion rates and reverse cumulative distributions should be provided. Seroconversion for polio antigen is defined as:

- for subjects seronegative at the pre-vaccination time point: antibody titres above the cut-off (titre 1/4–1/8);
- for subjects seropositive at the pre-vaccination timepoint: antibody titres that are fourfold the expected titre of maternal antibodies based on the pre-vaccination titre declining with a half-life of 28 days;
- in populations with high antibody titres: a change from below the highest dilution tested (<8192) to above the highest dilution tested (>8192) will also indicate seroconversion.

The half-life of maternal antibody decay may be assumed to be 28 days (52). It is desirable to consider these two parameters separately in the comparison between a new OPV and a licensed one used as control.

WHO has made an effort to standardize polio virology methods, leading to the publication in 1990 of WHO’s Manual for the virological investigation of polio (53). It is recommended that a standardized technique for measurement of neutralizing antibodies, involving standard cell lines and other standard reagents, should be used, such as International Standards of anti-poliovirus sera for types 1, 2 and 3, and that the results should be expressed in international units of neutralizing antibody (54, 55).

C.2.2 Immunogenicity studies

New candidate OPVs manufactured using different vaccine compositions (e.g. monovalent or bivalent) should be compared with a licensed formulation. New candidate vaccines should be compared to at least one well-established and licensed OPV. 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.

C.2.3 Population

The evaluation of new OPV formulations, including monovalent, bivalent and trivalent vaccines, based on Sabin strains may be conducted in infants and newborns since safety profiles in these populations have already been established.

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 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 (16).

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 OPV formulations (e.g. monovalent, bivalent and trivalent) contained the recommended dose for each poliovirus type (not less than $10^{6.0} \text{CCID}_{50}$ for type 1, $10^{5.0} \text{CCID}_{50}$ for type 2, and $10^{5.5} \text{CCID}_{50}$ for type 3). However, development of novel formulations with improved stability (addition of stabilizers/exipients) 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 (16). These data could also be used to support the derivation of 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 vaccine composition may impact virus replication in the intestinal tract and may influence the ability to induce an immune response. 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. Evaluation of virus excretion of new vaccine formulations containing one, two or three serotypes (i.e. monovalent, bivalent or trivalent) should be evaluated in comparison to the trivalent licensed formulation (18).

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 mOPV. 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 (50).

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 (16). 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. 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 national regulatory authorities

D.1 General

The general recommendations for national regulatory authorities and national control laboratories given in the Guidelines for national authorities on quality assurance for biological products (56) and Guidelines for independent lot release of vaccines by regulatory authorities (57) should apply.

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 International Standards currently in force should be obtained for the purpose of calibration of the national/regional/working standards (58). The NRA may obtain the product-specific/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 with regard to the neurovirulence test can be used by the manufacturer for the formulation of a final bulk.

If the national control laboratory does not perform the monkey neurovirulence test itself, it should carry out a second reading of the histological sections provided by the manufacturer for each monovalent bulk. In addition, the national control laboratory 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.

If the national control laboratory performs the mouse neurovirulence 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 days 3 and 4 plus day 14 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, 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 the present recommendations (57).

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

A statement signed by the appropriate official of the NRA should be provided to the manufacturing establishment and should certify that the lot of vaccine in question meets all national requirements, as well as Part A of these recommendations. The certificate should provide sufficient information on the vaccine lot. A model certificate is given in Appendix 6. The official national release certificate should be provided to importers of the vaccines. The purpose of the certificate is to facilitate the exchange of vaccines between countries.
Part E. Recommendations for live attenuated poliomyelitis vaccine (oral) prepared in primary cultures of monkey kidney

The following additional or alternative recommendations are for OPV prepared in cultures of primary monkey kidney cells and concern the testing of the cell substrate used for the production of the vaccine (Part A, section A.4). They should therefore be added to, or substituted for, the appropriate sections in Part A. Recommendations E.4.1, E.4.3.1.1, E.4.4.1 and E.4.4.2.1 are additions; recommendations E.4.2.1 and E.4.2.2 together replace A.4.2.1, and E.4.2.3 replaces A.4.1. All the other recommendations given in Parts A and B of the document are also applicable to this vaccine.

E.4 Control of vaccine production

E.4.1 Control of source materials

*E.4.1.1 Monkeys used for preparation of kidney-cell cultures and for testing of virus.*

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

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 groups\(^1\) 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 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.

\(^1\) 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.
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.4.2 Production precautions**

The general production precautions called for by the *Good manufacturing practices for biological products* (37) should apply to the manufacture of vaccine, with the addition of the following tests.

**E.4.2.1 Monkey kidney-cell cultures for vaccine production**

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.4.2.2 Tests of cell cultures used for vaccine production (see Appendix 7)**

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.¹ 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 cm² 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.

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.
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.4.2.3 Test of control cell cultures**
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 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.4.2.3.1 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.4.2.3.2 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.4.2.2.

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.4.2.2.
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.4.2.2, 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.4.3 Control of single harvests**

**E.4.3.1 Single harvest**

**E.4.3.1.1 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.4.4 Control of monovalent bulk**

**E.4.4.1 Monovalent bulk (before filtration)**

**E.4.4.1.1 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.

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.

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.

If the presence of B virus is demonstrated, the measures concerning vaccine production described in Part E, section E.4.2.2, should be taken.

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

**E.4.4.2 Monovalent bulk (after filtration)**

**E.4.4.2.1 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 (36).
Authors and acknowledgements

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Further changes were made to document WHO/BS/2012.2185 by the Expert Committee on Biological Standardization, resulting in the present document.

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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 current 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 (as of June 2012). They do not indicate any WHO "qualification" or "approval" of the strains or vaccines in the context of this document.
Figure 1. Types 1 and 2 OPV
Figure 2. Type 3 OPV
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**     Federal State Unitary Enterprise of Chumakov Institute of Poliomyelitis and Viral Encephalitides  
**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**

In vivo tests for neurovirulence and considerations for the choice of assay

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 poliovirus receptor (TgPVR21 mice) has been developed as an alternative to the MNVT for all three poliovirus serotypes.

Summaries of the MNVT and TgmNVT are given below, along with the implementation process for the TgmNVT.

1. Summary of the monkey neurovirulence test (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.\(^1\) 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. 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

\(^1\) Contact the Coordinator, Quality, Safety and Standards, World Health Organization, 1211, Geneva 27, Switzerland (http://www.who.int/biologicals/vaccines/en/).
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 $C_1$, $C_2$ and $C_3$. If the mean lesion score of the test vaccine is greater than that of the concurrently tested reference by more than $C_1$, the vaccine is not acceptable. If the test vaccine gives a higher score than the reference but the difference in scores lies between $C_1$ and $C_2$, the vaccine may be retested and the results pooled; if the difference for the pooled test results is greater than $C_3$, the vaccine fails.

The values for $C_1$, $C_2$ and $C_3$ 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 transgenic mouse neurovirulence test (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

1 Contact the Coordinator, Quality, Safety and Standards, World Health Organization, 1211, Geneva 27, Switzerland (http://www.who.int/biologicals/vaccines/en/).
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 sections A.3.2.4.2 and A.4.4.7.2 respectively.

4. Considerations for the choice of assay

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 (I).

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’ noncoding region 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 from the use of MNVT and TgmNVT with type 3 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.

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Appendix 3

Preparation of live attenuated poliomyelitis vaccine (oral) using cell banks
Example of a flowsheet of tests in cell cultures

* 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 (1).
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.
Reference

Appendix 4

Cell-culture techniques for the determination of the virus content of live attenuated poliomyelitis vaccine (oral)

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 $\log_{10}$ 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 antiserum (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 $\pm 0.5 \log_{10}$ CCID$_{50}$ 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 $\pm 0.3 \log_{10}$ CCID$_{50}$.

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 $\pm 0.3 \log_{10}$ CCID$_{50}$. 
Appendix 5

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

The following protocol is intended for guidance, and indicates the information that should be provided as a minimum by the manufacturer to the NRA.

Information and tests may be added or deleted as required by the NRA, if applicable. It is thus 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 given in the protocol submitted.

The section concerning the final lot must be accompanied by a sample of the label and a copy of the leaflet 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 of the country in which the vaccine was produced/released, stating that the product meets the national requirements as well as the recommendations of Part A of this document published by WHO.

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 suspensions blended in monovalent/bivalent/trivalent vaccine: | Type 1 | Type 2 | Type 3 |
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): ____________________________

Expiration 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: ____________________________

Starting 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 (section A.3.1.2)
Percentage of total cell-bank ampoules tested:

Identification of cell substrate:

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:

Volume of inoculum per vial:

Date of start of test:

Date of end of test:
**Result:**

**Tests for bacteria, fungi and mycoplasma**

*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>
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<td></td>
<td></td>
</tr>
<tr>
<td>Negative control:</td>
<td></td>
<td></td>
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</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>
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</tr>
<tr>
<td>Subcultures at day 7:</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Subcultures at day 14:</td>
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</tr>
<tr>
<td>Subcultures at day 21:</td>
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</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: __________________________________________________

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**

Date(s) of satisfactory test(s) for freedom from adventitious agent: ________________________________________

Volume of virus seed samples for neutralization and testing: ________________________________________

Batch number of antisera used for neutralization virus seed: ________________________________________

Method used: ________________________________________

Date of start of test: ________________________________________
Identity test

Method used:

Date of start of test:

Date of end of test:

Result:

Absence of SV40

Method used:

Date of start of test:

Date of end of test:

Results:

In vitro tests: MAPREC or rct/40 marker test

MAPREC

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

In vitro rct/40 marker test

Date of test:
Reduction of titre of bulk sample: ____________________________

Reduction of titre of negative reference: ____________________________

Reduction of titre of positive reference: ____________________________

Result: ____________________________________________

Result of test of consistency of production: ____________________________

**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 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:  

**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>
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<td></td>
</tr>
<tr>
<td>30–36 °C</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td></td>
<td></td>
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<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.4.1)_

_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 on supernatant culture 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)**

Volume harvested: ________________________________
Date of sampling: _______________________________________________________

**Identity test**
Method used: __________________________________________________________
Date of start of test: _____________________________________________________
Date of end of test: _______________________________________________________
Result: _________________________________________________________________

**Virus titration**
Date of test: _____________________________________________________________
Reference batch number: _________________________________________________
Date of test: _____________________________________________________________
Result: _________________________________________________________________

**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>Sub cultures at 7th day</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>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: ___________________________________________________

**Control of monovalent bulk (section A.4.4)**

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>
<tr>
<td>Negative control:</td>
<td>_____</td>
<td>_____</td>
<td>_____</td>
<td>_____</td>
<td>_____</td>
</tr>
</tbody>
</table>

**Test for mycobacteria**

Method used:  
Date of start of test:  
Date of end of test:  
Result:  

**Tests for consistency of virus characteristics**

**In vitro rct/40 marker test**

Date of test:  
Reference used:  
Reduction or titre of negative reference: ________________________________

Reduction of titre of positive reference: ________________________________

Result: ____________________________________________________________

MAPREC
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 % 472-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: ________________

Neurovirulence tests 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: 

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</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
volume of stabilizer: ______________ ______________ ______________
Nature and volume of diluent: ________________________________
Total volume of blend: ______________________________________

**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): ________________________________

<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>
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<td>________</td>
</tr>
<tr>
<td>30–36 °C</td>
<td>________</td>
<td>________</td>
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<td>________</td>
<td>________</td>
</tr>
<tr>
<td>Negative control</td>
<td>________</td>
<td>________</td>
<td>________</td>
<td>________</td>
<td>________</td>
</tr>
</tbody>
</table>

**Virus titration**

Date of test: ________________________________

Reference batch number: ________________________________

Titre of individual virus types: ________________________________

Batch numbers of antiserum used in test: ________________________________

Date of test: ________________________________

Results

<table>
<thead>
<tr>
<th></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: Vaccine at 37 °C  Vaccine  Difference

Total virus: ________  ________  ________
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: ________________________________

**Submission addressed to National Regulatory Authority**

Name of head of production (typed) ________________________________

_Certification by the person from the control laboratory of the manufacturing company taking overall responsibility for the production and 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 _Recommendations to assure the quality, safety and efficacy of live attenuated poliomyelitis vaccine (oral)._ 

Signature: ________________________________

Name (typed): ________________________________

Date: _____
Appendix 6

Model certificate for the release of live attenuated poliomyelitis vaccine (oral) by national regulatory authorities

Lot release certificate

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), Good manufacturing practices for biological products(7) and Guidelines for independent lot release of vaccines by regulatory authorities(8).

The release decision is based on _____________________________________________ (9).

The certificate may include the following information:
- Name and address of manufacturer
- Site(s) of manufacturing
- Trade name and 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/lot size
- Date of start of period of validity (e.g. manufacturing date) and/or expiry date
- Storage condition
- 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 National Regulatory Authority (or authority as appropriate):

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.
4 With the exception of provisions on distribution and shipping, which the NRA may not be in a position to assess.
8 WHO Guidelines for Independent Lot Release of Vaccines by Regulatory Authorities adopted by the Sixty-first meeting of the WHO Expert Committee on Biological Standardization in 2010.
9 Evaluation of summary protocol, independent laboratory testing, and/or specific procedures laid down in defined document etc., as appropriate.
Appendix 7

Preparation of live attenuated poliomyelitis vaccine (oral) using monkey kidney-cell cultures

Example of a flowsheet of tests in cell cultures

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.