# Chapter 15

# Methods for the Quality Control of Inactivated Poliovirus Vaccines

**Thomas Wilton**

## Abstract

Inactivated poliovirus vaccine (IPV) plays an instrumental role in the Global Poliovirus Eradication Initiative (GPEI). The quality of IPV is controlled by assessment of the potency of vaccine batches. The potency of IPV can be assessed by both in vivo and in vitro methods. In vitro potency assessment is based upon the assessment of the quantity of the D-Antigen (D-Ag) units in an IPV. The D-Ag unit is used as a measure of potency as it is largely expressed on native infectious virions and is the protective immunogen. The most commonly used in vitro test is the indirect ELISA which is used to ensure consistency throughout production.

A range of in vivo assays have been developed in monkeys, chicks, guinea pigs, mice, and rats to assess the potency of IPV. All are based on assessment of the neutralizing antibody titer within the sera of the respective animal model. The rat potency test has become the favored in vivo potency test as it shows minimal variation between laboratories and the antibody patterns of rats and humans are similar. With the development of transgenic mice expressing the human poliovirus receptor, immunization-challenge tests have been developed to assess the potency of IPVs. This chapter describes in detail the methodology of these three laboratory tests to assess the quality of IPVs.

**Key words** ELISA, Rat potency, Neutralization titer, Back titration, Transgenic mice, Human poliovirus receptor, Immunization-challenge, Biosensor, Surface plasmon resonance

## 1 Introduction

In the late nineteenth century improvements in hygiene standards in some countries delayed transmission of poliovirus in new born infants until maternal antibodies had waned. This delay in transmission consequently changed the pattern of poliomyelitis in those countries from endemic to epidemic. The occurrence of epidemics involving large number of children, many of whom became paralyzed, prompted a research based response focussed on developing effective vaccines against poliovirus. It was demonstrated that poliovirus could be propagated in human cells in culture [1], as well as a range of cells from tissues of nonhuman primates. The monkey kidney became the source of tissue for vaccine

Javier Martín (ed.), *Poliovirus: Methods and Protocols*, Methods in Molecular Biology, vol. 1387,
DOI 10.1007/978-1-4939-3292-4_15, 

production, allowing the development of two vaccines; an injectable (killed) inactivated poliovirus vaccine (IPV) by Salk et al. [2], and a live attenuated oral poliovirus vaccine (OPV) by Sabin [3]. This chapter focuses on IPV and the methods used to assess the quality of the vaccine.

IPV is a mixture of three PV strains, one from each of the three serotype, made by harvesting cell culture supernatants, purifying and inactivating them using formaldehyde. In the early 1950s, following several technical developments, Salk and colleagues were able to grow large quantities of the three PV serotypes and analyze the kinetics of inactivation with formaldehyde (HCHO) [4]. It was concluded that if filtration was used to remove aggregates of PV, the virus could be inactivated at a constant rate, allowing complete elimination of infectivity provided that sufficient time was allowed for complete inactivation. Several trivalent vaccine pools were prepared and found to be safe and immunogenic in both monkeys and humans [5], based on these laboratory findings large-scale field trials in humans were conducted by Thomas Francis and his associates in 1954. The vaccine was found to be safe and effective at 60–70 % for serotype 1 and 90 % for serotype 2 and 3. Potency, based on the antibody response in children, correlated with effectiveness of the vaccine [6] and IPV was licensed shortly after.

Following several technical advances in the late 1960s and 1970s, enhanced-potency IPV (eIPV) was developed. This second generation IPV differed from the first generation IPV as the virus harvest was concentrated and purified before inactivation by ultrafiltration and column chromatography. In addition, to improve virus yield, they increased the density of cells by growing them on microbeads in large fermenters. In 1967 van Wezel developed a microcarrier system which could be applied to 100 l fermenters [7], either secondary or tertiary subcultures of kidneys from pathogen-free monkeys or human diploid cell strains or the Vero African green monkey kidney cell line were used as the cell substrate. The use of well characterized cells ensures that IPV is free of extraneous contaminating agents. This trivalent eIPV acts as the conventional IPV (cIPV) and is administered either alone or in combination with other vaccines, including diphtheria, tetanus and acellular pertussis, hepatitis B and/or Hemophilus influenzae b. These IPV preparations are highly immunogenic in infants following three doses at 2, 3, and 4 months of age [5].

The wild-type PV strains used by Salk et al. for the first generation IPV are still used by most modern manufacturers. These include Mahoney (serotype 1; Brunenders is used in Sweden and Denmark); MEF-1 (serotype 2); and Saukett (serotype 3). Although HCHO inactivation has been noted to modify the antigenic site 1 of PV serotypes 2 and 3 [8], immunization with IPV can induce high titers of neutralizing antibodies protective against all PV strains.

One of the key means for the quality control of IPV is the assessment of the immunogenicity or potency of the vaccine. When the IPV was developed by Salk in the 1950s, the potency of IPVs was not assessed; instead each dose of IPV was designed to be the equivalent of a specific volume of harvest fluid from PV-infected primary monkey kidney tissue cells. Inactivated poliovirus vaccines assessed by this method showed variable immunogenicity in humans. In response to the Cutter incident in which vaccine recipients were paralyzed by the use of incompletely inactivated IPV, filtration steps were introduced to remove aggregates [9]. While these steps led to increased safety, the antigenicity of the IPVs fell as a result and this, in turn, lowered the immunogenicity of the vaccine. Consequently, potency assays for the final products were required.

Both in vitro and in vivo potency assays have been developed to assess the immunogenicity of IPV. Previously the in vivo assays were used as the official batch release tests carried out on the final IPV product, while the in vitro assays were principally used for in-process monitoring. This situation has since changed and due to the requirements of the European convention on the protection of vertebrate animals used for experimental and other scientific purposes, it is possible to waive the in vivo assay and assess the potency solely by in vitro assays, should certain conditions be met [10]. In vitro assays measure the potency of IPV preparations by D-Ag units. In the 1960s the D-Ag and C-Ag units were established following characterization of purified virus preparations by sucrose gradient centrifugation where two bands were identified [11]. The D-Ag unit is used as a measure of potency as it is largely expressed on native infectious virions and is the protective immunogen. The most commonly used in vitro test is the indirect ELISA. This assay was first developed in 1980 and replaced the gel diffusion method as it was found to be more sensitive and less time consuming [12]. It is used to assess the D-Ag content of IPVs and ensure consistency throughout production. This assay is described in detail below.

A range of in vivo assays have been developed in monkeys, chicks, guinea pigs, mice and rats. All are based on assessment of the neutralizing antibody titer within the sera of the respective animal model. Initially the monkey potency test was the standard in vivo assay to determine the potency of IPV preparations [13]. In this test at least ten simians are inoculated, using a three dose schedule comparable to that used for human recipients. The geometric mean titer of neutralizing antibodies for each of the three serotypes is compared with a reference trivalent antiserum. An IPV is deemed acceptable if the mean titer is above that of the reference antiserum.

While the monkey potency test can distinguish qualitatively between good and poor IPVs, it is not sufficient for obtaining quantitative data about vaccine potency, as it uses a reference serum

rather than a reference vaccine. In addition, this test requires many expensive primates. These concerns led to a search for another animal model to assess the potency of IPVs. Animals which are nonsusceptible to PV are known to be capable of forming specific neutralizing antibodies following parenteral administration of live or inactivated PV. Consequently, a range of nonsusceptible animal models have been developed, two of which are the guinea pig/chick models [14, 15]. These two models are based on a potency tests in which the extinction end-point of the median effective dose (ED50) is calculated after the animals have been inoculated with a single dose of the vaccine. Both the guinea pig and chick potency test have been adopted as a single test within the European Pharmacopoeia. In this test, three or more dilutions of an IPV are used to immunize either guinea pigs or 3-week-old chicks (ten animals/dilution). After 5–6 days, animals are bled and sera are diluted to one in four. Poliovirus (100 TCID50) is mixed with the diluted serum and incubated at 37 °C for 4.5–6 h and then 5 ± 3 °C for 12–18 h. Mixtures are added to cell cultures for up to 7 days, to detect unneutralized PV. For each group of animals, the number of sera which have neutralizing antibodies is noted and the dilution of the IPV which gives an antibody response in 50 % of the animals (the end-point of the ED50) is calculated. An IPV is acceptable if a dilution of 1 in 100 or more produces an antibody response for each of the three serotypes of PV in 50 % of the animals.

Although an accepted technique of the European Pharmacopoeia, the guinea pig/chick potency test is limited in that the calculated single dose ED50 can only provide a qualitative measure of the immunogenicity of an IPV. This test lacks the sensitivity and reproducibility to distinguish between IPVs which differ in antigenic content as much as fourfold to eightfold. In addition, this test is a poor predictor of human immune response. In particular, this test can only measure IgM instead of IgG which is typically measured following vaccination in humans. In response to these limitations the Rijks Instituut voor de Volksgezondheid developed an alternative potency test in which the titer of the antibody response was measured rather than the extinction titer (end-point). The antibody response of guinea pigs and various strains of rats and mice were assessed to identify the animal which would produce a good dose-related titer response after a single injection, preferably in the IgG class. Rats were found to give the highest titers, a good dose-related titer response in the IgG class and were consistent across different strains. Consequently, a rat potency test was developed to assess the immunogenicity of a range of IPVs in relation to a reference IPV of proven efficacy in humans. Comparable distribution of antibody titers for reference and sample IPVs was found, allowing accurate assessment of potency. Antibody patterns of rats and humans were similar, indicating that the rat maybe a suitable

model to assess the immunogenicity of IPV in humans [16]. An international collaborative study compared the use of the chick/guinea pig and the rat potency tests to determine the immunogenicity of six trivalent IPVs. Wide variation was found between laboratories using the chick/guinea pig potency test. The rat potency test was found to be far less variable between laboratories [17]. The results of this study led to the validation of the rat potency test by manufacturers and national control laboratories as an alternative means of determining the immunogenicity of IPVs. The rat potency test is now included within the European Pharmacopoeia [18]. Currently the European Pharmacopoeia guidelines require that the potency of an IPV is assessed either in vivo by the chick/guinea pig or rat tests or by an in vitro method (following a waiving of in vivo tests). Due to its benefits, the rat potency test is considered the in vivo method of choice. The rat potency test is described in detail below.

Recently an immunization-challenge test using transgenic (Tg) mice to further evaluate the immunogenicity and protective properties of IPVs has been developed independently by the US Food and Drug Administration (FDA) and NIBSC [19, 20]. The identification and isolation of genes which encode human and primate PVRs has allowed Tg mice lines which express the human PVR (TgPVR mice) to be established. Transgenic mice expressing the human PVR can be infected with PV serotypes by various routes and develop clinical signs of paralysis and morphological lesions in the CNS. As the TgPVR mice can clinically manifest paralysis, it is possible to determine the 50 % end-points for paralysis ($PD_{50}$) or lethality ($LD_{50}$), if a suitable dose range of a PV is used.

Previous research by the FDA and the NIBSC has identified suitable immunization-challenge regimes with TgPVR mice for assessing the immunogenicity of IPV preparations. A single or booster vaccination (1 week apart) followed by a challenge was sufficient to model protection. In Tg21PVR mice both protection and the level of neutralizing antibody elicited by vaccination with IPV have been found to be dose-dependent. A good correlation has been found between the neutralizing antibody titers in blood samples from Tg21PVR mice and the immune protection conferred. Immunization-challenge regimes with TgPVR mice allow a more direct analysis of the protection conferred by IPV preparations than other potency test which assess the immunogenicity on the basis of seroconversion. The immunization-challenge test with TgPVR mice assesses the protection conferred by other aspects of the immune response (e.g., cellular immunity) in addition to the neutralizing antibody response. The immunization-challenge assay with TgPVR mice is described in detail below.

## 2 Materials

### 2.1 ELISA Potency Test

#### 2.1.1 Equipment

- +4 °C refrigerator.
- –20 °C freezer.
- –70 °C freezer.
- Incubator set at 37 °C.
- 96-well microtiter plates for ELISA.
- 2 ml microtubes.
- Pipettors in calibration.
- Multichannel pipette and suitable tips.
- Sterile 1, 5, 10, and 25 ml serological pipettes.
- Multipipette and tips.
- Multidrop Combi Reagent Dispenser.
- Spectrophotometer for 96-well plates with 492 nm (nominal) filter.
- Balance.
- Plastic box.
- Vortex mixer.

#### 2.1.2 Reagents

- Test vaccines—stored at either 2–8 °C (refrigerator) or –20 °C or –70 °C (Freezer).
- Dulbecco's 6 salt PBS.
- Dried milk powder.
- Tween 20.
- Carbonate coating buffer (storage at 2–8 °C)—6.36 g sodium carbonate and 11.72 g sodium hydrogen carbonate made up to 4 l with deionised $H_2O$.
- Wash Buffer—Dulbecco's 6 Salt PBS containing 2.0 % dried milk and 0.5 % Tween 20—prepare on day of assay and discard any unused buffer after use.
- Assay diluent—Dulbecco's 6 Salt PBS containing 2.0 % dried milk powder—prepare on day of assay and discard any unused buffer after use.
- Serotype specific capture antisera (*see* **Note 1**).
- Monoclonal antibodies (MAbs) (*see* **Note 1**).
- Reference vaccine (with assigned potency in D-Ag units). Any reference vaccine used in the study should have been calibrated using an International Standard for IPV. The current WHO International Standard (12/104) has an assigned potency of 277, 65 and 248 D-Ag units for types 1, 2 and 3 respectively (stored at –70 °C). The current European Pharmacopoeia biological reference preparation (Ph Eur BRP Batch 2) has an

assigned potency of 320-67-282 D-Ag units for types 1, 2 and 3 respectively.

- Monitor vaccine samples.

A previously validated monitor IPV sample must be included in all batch release assays.

- Peroxidase conjugated anti-mouse IgG whole molecule (Sigma-Aldrich, store at –20 °C) diluted in assay diluent to a working dilution of 1:400.
- Substrate buffer—Mix 12.15 ml 0.1 M citric acid, 12.85 ml 0.2 M $Na_2HPO_4$, and 25 ml distilled $H_2O$. Prepare immediately before use.
- Substrate reagents:

0.1 M citric acid—19.2 g made up to 1 l with distilled $H_2O$ in a measuring cylinder or

0.1 M citric acid·$H_2O$—21.0 g made up to 1 l with distilled $H_2O$ in a measuring cylinder (Storage—room temperature).

0.2 M $Na_2HPO_4$—28.4 g made up to 1 l with distilled $H_2O$ in a measuring cylinder or

0.2 M $Na_2HPO_4 \cdot 12H_2O$—71.63 g made up to 1 l with distilled $H_2O$ in a measuring cylinder (Storage—room temperature).

- OPD substrate—Prepare in a 50 ml centrifuge tube: 1 × 30 mg *o*-phenylenediamine dihydrochloride substrate tablet (Sigma-Aldrich) + 50 ml substrate buffer + 50 µl hydrogen peroxide (30 %, Sigma-Aldrich). Use within 1 h of addition of tablet to buffer and add $H_2O_2$ immediately before use (Store in dark).
- Stop solution—1 M $H_2SO_4$ (in the proportion of 1 ml concentrated acid in 17 ml $H_2O$) (Storage—room temperature).

## 2.2 Rat Potency Test

### 2.2.1 Equipment

- Class II Microbiological safety cabinet.
- Refrigerated centrifuge.
- Sterile universal bottles.
- +4 °C refrigerator (+2–8 °C).
- –20 °C freezer.
- –80 °C freezer.
- Multipipette and sterile tips.
- Half test tubes in racks.
- Sterile 1, 5, 10 and 25 ml pipettes.
- Microtiter plates (flat bottomed sterile tissue culture grade).
- Pressure sensitive film (PSF).
- Sterile blotting paper.
- Multidrop Combi Reagent Dispenser.
- Inverted microscope.

- Incubator at +35 + 1 °C.
- Gilson and electronic pipettes in calibration + sterile tips.
- Multichannel pipettes in calibration + sterile tips.
- Sterile Multidrop dispensing cassettes.

### 2.2.2 Reagents

- Assay diluent for inoculum to be immunized to rats (100 ml volume):

| | |
|---|---|
| Eagles Minimum Essential Medium (MEM) 10× concentration | 10 ml |
| $NaHCO_3$ (7.5 %) (Sigma-Aldrich) | 2.9 ml |
| 7 % Bovine albumin in MEM | 2 ml |
| Sterile distilled water | Up to 100 ml |

Assay diluent is made up before use using Class II Microbiological safety cabinet and used immediately without storage.

- Assay diluent for neutralization assay (500 ml volume):

| | |
|---|---|
| MEM ×1 concentration | Up to 500 ml |
| Or MEM 10 concentration | 50 ml |
| $NaHCO_3$ (7.5 %) (Only required with ×10 MEM) | 8.85 ml |
| Penicillin + Streptomycin (Sigma-Aldrich) | 5 ml |
| (With 10,000 units' penicillin and 10 mg streptomycin/ml in 0.9 % NaCl) | |
| Amphotericin B solution (250 μg/ml) (Sigma-Aldrich) | 5 ml |
| Fetal calf serum | 20 ml |
| l-Glutamine (200 mM) (Sigma-Aldrich) | 5 ml |
| Sterile water (only required with ×10 MEM) | Make up to 500 ml |

- Assay diluent is made up before use and used immediately without storage.
- 0.25 % trypsin—EDTA solution (Sigma-Aldrich).
- HEp2-C cells. Use HEp2-C 75 $cm^3$ flask stock cultures up to 7 days from the last subculture. (No maximum passage level has been established for this assay method however cells are not used for more than 3 months before returning to original stocks.)
- Plastic stain bath containing naphthalene black—6 % acetic acid (100 % glacial), 1.3 % sodium acetate, 1 % naphthalene black.

- Reference vaccine.

  A previously validated vaccine reference has to be used in the assay. Ideally, this reference should be traceable to clinical studies showing suitable immunogenicity in humans. An established International Standard for IPV should be used to validate the assay.
- Monovalent reference polioviruses for each serotype.
- In-house reference positive and negative sera of known neutralization titer.

### *2.3 Immunization-challenge assay in TgPVR mice.*

#### *2.3.1 Equipment*

- Class II microbiological safety cabinet.
- +4 °C refrigerator (+2-8 °C).
- –20 °C freezer.
- –80 °C freezer.
- 2 ml microtubes.
- Gilson and electronic pipettes in calibration.
- Sterile 1, 5, 10, and 25 ml serological pipettes.
- Vortex.
- All equipment needed to house, handle, and inoculate TgPVR mice is not listed here.

#### *2.3.2 Reagents*

- Transgenic mice expressing the human poliovirus receptor so they are susceptible for poliovirus infection and paralysis.
- Eagles Minimum Essential Medium (MEM) 1× concentration is used as assay diluent to prepare solutions containing vaccines and virus challenges Assay diluent is handled in a sterile manner in a Class II microbiological safety cabinet and used immediately without storage.
- Reference vaccine.

  A previously validated vaccine reference has to be used in the assay. Ideally, this reference should be traceable to clinical studies showing suitable immunogenicity in humans. An established International Standard for IPV should be used to validate the assay.

## 3 Methods

### *3.1 ELISA Potency Test*

#### *3.1.1 Preparation of Assay*

1. In a class II safety cabinet, dilute serotype specific capture antibody in carbonate coating buffer as required (*see* **Note 1**). Add 50 µl to each well of a 96-well ELISA plate. One plate per poliovirus serotype.
2. Incubate overnight at 2–8 °C in a box with a humidified atmosphere.

3. Wash each ELISA plate 4× with wash buffer. Leave plate containing last wash at room temperature for at least 30 min.
4. Prepare independent series of 4, twofold dilutions of reference and test vaccines in assay diluent.
5. Start at 1 in 20 for the IRR and BRP. For non-desorbed single human dose vaccines (40:8:32, respective virus types) use a starting dilution of 1 in 3. Predilute vaccines of different formulations to approximately this concentration in assay diluent.
6. Add 50 µl of vaccine or reference dilution to a minimum of 2 wells per dilution for each independent series of dilutions. Add diluent only to row 1 and 12 to serve as a blank.
7. Seal plates and incubate for at least 2 h at 35–38 °C in a box with a humidified atmosphere.
8. Wash 3× with wash buffer.
9. Add 50 µl of monoclonal antibody (*see* **Note 1**) diluted in assay diluent to each well, including blanks.
10. Seal plates and incubate for at least 1 h but not more than 75 min at 35–38 °C in a box with a humidified atmosphere.
11. Wash 3× with wash buffer.
12. Add 50 µl peroxidase conjugated anti-mouse diluted in assay diluent to each well.
13. Seal plates and incubate for at least 1 h but not more than 75 min at 35–38 °C in a box with a humidified atmosphere.
14. Wash 3× with PBS.
15. Add 50 µl OPD substrate to each well. Leave at room temperature in the dark.
16. Stop reaction after 30 min by addition of 50 µl of 1 M $H_2SO_4$ per well.
17. Read optical density at 492 nm as soon as possible.

### 3.1.2 Analysis of Results

1. Parallel line analysis is performed on the assay data by using a statistical program such as CombiStats. This program gives the potency of the test vaccine relative to the concurrently tested reference. The dilutions and the corresponding OD values are used to calculate the D-Ag content of each vaccine relative to the reference (*see* **Note 1**).
2. For an assay to be valid the dose response curves should be linear and parallel. A minimum of three dilutions on the linear section of the dose response curve should be used in the analysis. Tests for linearity and parallelism are performed by the computer program.

3. The levels of significance of deviation from linearity and parallelism for the reference, monitor and all individual vaccine samples should be 1 % or below.
4. The D-Ag content of the monitor sample should be within specifications assigned for that sample.

#### 3.1.3 Interpretation of Results

Vaccines tested in assays that meet all the assay validity criteria should be assessed with regard to their D-Ag content/dose. Vaccines must have D-Ag within the given release specification for a vaccine to be recommended for release. Recent research has been directed to assessing alternative methods to the current ELISA protocol for establishing the D-Ag content of IPV (*see* **Note 2**).

### 3.2 Rat Potency Test

This potency test involves inoculating trivalent test vaccine/s plus a reference into rats (*see* **Note 3**). The rats are bled between 20 and 22 days and the sera is then tested for neutralizing antibody to all three polio virus types using a fixed virus varying serum, 50 % end point technique in a microtiter system. A vaccine is satisfactory if the potency is not significantly less than that of the reference preparation. This testing is undertaken in line with European Pharmacopoeia (Ph Eur) potency test for IPV.

#### 3.2.1 Inoculation of Rats

1. Prepare assay diluent for inoculation of rats using a class II cabinet.
2. Remove the vaccines from +4 °C and the appropriate reference preparation from either −80 °C or +4 °C.
3. Assess the appearance of the vaccine (described in the product licence). Assess the sample against a light background with light coming from the side and also against a matt black background. This is to ensure it meets manufacturer's specifications and does not contain any extraneous particles. If the vaccine fails to meet the criteria then the test is stopped.
4. Label sterile universal bottles with the sample name, test number, dilution, and test date. Transfer of sample from the original container to the first container of the dilution series is witnessed and countersigned by a second person.
5. Prepare dilutions of test vaccine and reference as detailed in Table 1.
6. Inoculate each group of rats with $2 \times 0.25$ ml of diluted material into each hind leg.
7. Following 20–22 days post-inoculation anesthetize rats and bleed out by cardiac puncture.
8. Allow blood samples to clot at +4 °C for 1–2 days.
9. Centrifuge all blood samples at $800–1000 \times g$ in a refrigerated centrifuge for 5 min to aid separation of the blood clot and sera.

**Table 1**
**Preparation of dilutions of test vaccine and pre-diluted reference**

| | Dilution | Volume diluent | Vol. sample (ml) | No. of rats |
|---|---|---|---|---|
| Vaccine + reference | Undiluted | NA | 12 | 10 |
| | 1/2 | 6 ml | 6 | 10 |
| | 1/4 | 6 ml | 6 | 10 |
| | 1/16 | 6 ml | 2 | 10 |
| Ref EP BRP or IS 91/574 | [a](See below) | 12 ml | 3 | 10 |
| | 1/2 | 6 ml | 6 | 10 |
| | 1/4 | 6 ml | 6 | 10 |
| | 1/16 | 6 ml | 2 | 10 |

[a]The first dilution made is in fact a 1:5 for these references. This dilution is required to bring the references to single human dose as they are 10× more concentration than the test vaccine. This dilution will be treated as undiluted as the reference now equals a single human dose as per the vaccine section

10. Transfer sera in a sterile manner to tubes labeled with the test number, rat cage number, a unique letter and the date.
11. Store the sera at –20 or –80 °C until required for testing.

### *3.2.2 Neutralizing Antibody Assay*

#### Preparation of Assay

1. Obtain a culture of Hep-2C cells. Check the culture microscopically for condition of monolayer and absence of overt bacterial contamination. Use only cultures which are confluent and in good condition.
2. Remove rat sera from –20/–80 °C storage and thaw overnight at +4 °C.
3. Thaw positive/negative reference sera from –20 °C overnight at +4 °C.
4. Prepare sufficient assay diluent for the test in the class II cabinet.
5. Prepare a worksheet in which code number assigned to each serum are recorded. Note the volumes used to make up the challenge virus on the worksheet.
6. Label all tubes and plates with the serum or sample number or the uniquely assigned code letter or number from the worksheet.

#### Dilution of Challenge Viruses to 100 Tissue Culture Infective Dose 50 % End point ($TCID_{50}$)/0.05 ml

1. Remove relevant challenge virus strains from –80 °C storage and thaw on ice.
2. Dilute the challenge virus strains to 100 Tissue Culture Infective Dose 50 % end point ($TCID_{50}$)/0.05 ml. This dilution is based on previously established titer of the challenge virus strains. The dilution to a challenge dose of 100 $TCID_{50}$/0.05 ml is done in a series of steps.

3. Adjust the volume of the last dilution step so that it is sufficient for the relevant number of plates.
4. Incubate 1.0 ml of each virus challenge with 1.0 ml of diluent in half test tubes in the same test conditions as the test plates to perform the back-titration of the virus challenge.

Titration of Sera

1. Prepare sufficient plates for the number of sera to be tested. Each serum will be added to three plates so that a different plate is used for each virus type.
2. A different starting dilution might be required for each serotype based on preliminary tests, label all plates with the serum number or unique code, the virus type and the assay date.
3. Use the plate in the 12 across 8 down orientation allowing six sera to be titrated per plate using this layout.
4. Three further plates will be needed for the in-house rat positive sera and one plate (or 6 wells) is needed for the in-house rat negative sera.
5. Using Titertek Multidrop dispensing machine dispense 0.05 ml of diluent into each well of a microtiter plate. Keep each plates covered as much as possible during manipulations using sterile blotting paper.
6. Add 0.05 ml of the first serum sample to columns 1 and 2 in Row A of each of three plates. Because an equal volume of medium has been added to each well.
7. Add 0.05 ml of the second serum to columns 3 and 4 in Row A of each of three plates and repeat this process for other serum samples until all samples are added.
8. Add 0.05 ml of the in-house rat negative sera to 2 wells per virus type. This can either be on a separate plate or at the end of the test sera.
9. Make a 1:1 dilution of the in-house rat positive sera by adding 1 ml of assay media to the 1 ml aliquot (so starting dilution on plate of 1/4). Then add 0.05 ml of the in-house rat positive sera to wells A-H in row 1 (this plate is in the 8–12 orientation). One plate will be used per type (8 wells per virus type).
10. Make twofold serial dilutions of all samples using a multichannel pipette with disposable tips. Do this by aspirating manually or electronically to mix and withdraw 0.05 ml volumes from each well in Row A and transfer these to Row B. Continue through to Row H and discard the last 0.05 ml.

Addition of Virus Challenge and Neutralization Step

1. Add 0.05 ml of each virus challenge to the appropriate plates using a suitable piece of equipment that is calibrated to dispense 50 μl.
2. Seal all plates with pressure sensitive film (PSF).

3. Incubate the assay, including the 1 + 1 virus challenge, overnight at +4 °C then for 3 h at 35 °C.

### Back Titration of Virus Challenge

1. After incubation, dispense 4.5 ml of diluent into 3 half test tubes for each virus type. Back titrate each 1 + 1 virus challenge sample a further 3× tenfold steps.
2. Transfer and mix 0.5 ml of 1 + 1 virus challenge into $10^{-1}$ tube. Use a fresh sterile 1 ml pipette or tip each time. Continue serial dilution for $10^{-2}$ and $10^{-3}$.
3. Inoculate 0.1 ml of each titration into eight wells of a microtiter plate beginning at most dilute and ending with the most concentrated dilution.
4. Repeat for each virus challenge.

### Addition of HEp2-C Cells

1. Prepare sufficient HEp-2C cell suspension for the number of plates in the assay, (10 ml of cell suspension per plate plus about 10 % extra) in class II safety cabinet as follows:
   (a) Decant medium from 75 $cm^2$ culture flasks.
   (b) Wash cell monolayer with PBS.
   (c) Add 5 ml of 0.25 % trypsin-EDTA solution.
   (d) Spread this over the cell sheet and decant.
   (e) Place culture at 35 °C for 5 min.
   (f) When cells begin to detach from the flask suspend in approx. 10 ml of diluent, aspirating well.
   (g) Make up to 100 ml using assay diluent. The cell suspension is now ready for use (approx cell count $1–2 \times 10^5$/ml).
2. Remove plates from the 35 °C incubator and remove PSF. Keep each plate covered with sterile blotting paper.
3. Using Titertek Multidrop dispensing machine add 0.1 ml of cell suspension into each well in all plates. Seal the plates thoroughly with PSF. Incubate the plates at 35 °C for 5–7 days. Ensure at least two rows or columns of cell controls are included in each test.
4. After incubation check plates visually for condition of cells and for absence of bacterial contamination. If contamination is suspected plates are read microscopically.
5. Uncontaminated plates are then fixed and stained as below and results are recorded. Contaminated plates are read microscopically by eye.

### Fixing and Staining

1. Remove pressure sensitive film from plates.
2. Discard medium from wells into 5 % Microsol.

3. Immerse plates in stain bath containing naphthalene black stain. Leave for at least 1 h or overnight.
4. Rinse in tap water, drain and dry in incubator.
5. Only those wells with a confluent monolayer of cells will be stained blue. These are scored negative. Those wells where polio cytopathic effect (CPE) has completely destroyed the monolayer will remain colorless. These are scored positive.

Assessment of the Assay

1. The test must be assessed for tissue condition: the cell controls must be in such condition that clear distinction can be made between the presence and absence of polio CPE. Negative wells on the test plates may also be used as guide to tissue condition.
2. Serum titrations with significant incidental losses of more than three wells must be repeated.
3. The titer of the challenge virus must be in the range 1.0–3.0 log10 TCID50.

Analysis of Results

1. The serum antibody titer is calculated as the highest dilution which protects 50 % of the cultures against 100 $TCID_{50}$ of challenge virus.
2. The raw data from the rat assays are converted to animals responding or not responding. The number of animals positive for neutralizing antibody out of the number inoculated is scored for both test vaccine and reference at each dilution for each of the polio virus types. The cut off values for assessing whether a serum is positive should be established before to define the dilution with best dynamic range. These are usually different for each of the three poliovirus serotypes. The geometric mean neutralization titers generated by each of the vaccines against different poliovirus challenge strains can also be determined and compared.
3. The number of animals positive for neutralizing antibody out of the number inoculated is scored for both test vaccine and reference at each dilution for each of the polio virus types. These results are used for determining if the response to test vaccine/s is significantly different to that of the reference vaccine.
4. The dose response for each preparation should be linear, and the dose-responses of the reference and test preparations should be parallel. Tests for linearity and parallelism are performed by computer analysis.
5. A potency value with confidence intervals is obtained for each test vaccine relative to the reference vaccine.
6. The "Upper limit" and "Lower limit" ($P = 0.950$) for vaccine potency must be within 25–400 % of the value.

7. The 50 % effective dose (ED50) is calculated and must lie between the largest and the smallest doses for both the reference and the test preparation for the assay to be valid.

Interpretation of Results

A vaccine is considered suitable for release if the potency of each of the polio types is not significantly less than that of the reference preparation. The release criteria is that the upper fiducial limit (UFL) of the relative potency for the vaccine must be greater or equal to 1.0 (>1.0). This specification is noted in the Ph Eur monograph for IPV.

### *3.3 Immunization-Challenge Assay in TgPVR Mice*

#### *3.3.1 Inoculation of TgPVR Mice*

1. Prepare assay diluent for inoculation of TgPVR mice using a class II cabinet.
2. Remove the vaccines from either –80 °C or +4 °C.
3. Assess the appearance of the vaccine (described in the product licence). Assess the sample against a light background with light coming from the side and also against a matt black background. This is to ensure it meets manufacturer's specifications and does not contain any extraneous particles. If the vaccine fails to meet the criteria then the test is stopped.
4. Label sterile bijou with the sample name, test number, and test date.
5. Prepare dilutions of test vaccine/s and reference to the required D-Ag dosage concentrations.
6. Inoculate groups of eight TgPVR mice of equivalent age and gender (6–8 weeks) by the intra-peritoneal route (0. 2 ml per mouse) with each test/reference vaccine or diluent medium.
7. Following 14 days, booster the mice with the same dose by the same route.
8. After a further 21 days tail bleed all mice.
9. Allow blood samples to clot at +4 °C for 1–2 days.
10. Centrifuge all blood samples at 800–1000 × *g* in a refrigerated centrifuge for 5 min to aid separation of the blood clot and sera.
11. Transfer sera in a sterile manner to tubes labeled with the test number, TgPVR mice cage number, a unique letter and the date.
12. Store the sera at –20 or –80 °C until required for testing. The sera can be tested for neutralization titers against different poliovirus strains following a neutralization assay similar to the one used for rat sera described above.
13. After obtaining tail bleeds prepare the virus inoculum containing the selected challenge PV strain at a dosage 10–50 × $PD_{50}$/0.05 ml to inoculate each TgPVR mouse.

14. Inoculate the mice by the intramuscular route with a paralyzing dose of the selected challenge PV strain.
15. Monitor the mice for signs of paralysis for 14 days. Clinically score daily— a minimum of partial paralysis in either hind limb must be apparent before mice are culled.

#### 3.3.2 Analysis of Results

1. The number of paralyzed mice is recorded for each group of mice.
2. For the assay to be valid, a significant number of animals in the control group (mock-immunized) should be paralyzed.
3. Survival curves showing the proportion of surviving animals during the 14-day observation period can be obtained using specialized software.
4. Statistical tests such as the Log-rank (Mantel–Cox) Test or the Gehan–Breslow–Wilcoxon Test can be used to determine if there are significant differences between survival curves of animal groups immunized with different vaccines and/or challenged with different poliovirus strains.

#### 3.3.3 Interpretation of Results

This assay allows comparison between vaccines for protection against challenge with paralytic poliovirus. It will also be possible to assess whether different vaccines protect differently against various poliovirus strains such as vaccine, wild or vaccine-derived isolates. These results should be compared to those measuring the neutralization antibody titers against different poliovirus strains sera from mice prior to the virus challenge.

## 4 Notes

1. A variety of combinations of capture-detection antibodies are used in different laboratories. These include both monoclonal and polyclonal antibodies obtained in different animals and used for either step and in all possible combinations. The use of such different platforms in different laboratories highlights the need for International Standards to calibrate laboratory references used in these assays. It is critical to calibrate any antibody reagents to specifically detect D-Ag potency.
2. One such method is the biosensor-based analytical system which is based on Surface Plasmon Resonance (SPR) technology. Surface Plasmon Resonance technology is an optical method which measures the refractive index of very thin layers of material adsorbed on a metal (such as gold). A detailed explanation of SPR technology can be found elsewhere [21, 22]. The SPR technology has been developed by various research institutions to study molecular interactions, including

GE Healthcare which has developed the Biacore biosensor. Preliminary work by Kersten et al. [23] using the Biacore biosensor indicates that the biosensor approach could be used to determine the D-Ag content of IPV. More recent research [24] has developed a biosensor-based protocol which established comparable D-Ag/ml estimates to those obtained by the current ELISA method, indicating that it could offer an alternative means to assess the potency of IPVs. Variation within assays and laboratories could be lessened by the automated nature of biosensors. The variability in polyclonal and monoclonal antibodies could be eliminated with the use of a biosensor system, as this would allow the rapid characterization (within days) of antibodies, which would enable laboratories to rapidly screen and select the optimal antibodies.

3. A good titer response has been found in both outbred (such as Wistar) and inbred strains (including Sprague Dawley, Lewis, and others). There is little difference in immune response between the rat strains [16].

## References


1. Enders JF, Weller TH, Robbins FC (1949) Cultivation of the Lansing strain of poliomyelitis virus in cultures of various human embryonic tissues. Science 109(2822):85–87
2. Salk JE, Krech U, Youngner JS, Bennett BL, Lewis LJ, Bazeley PL (1954) Formaldehyde treatment and safety testing of experimental poliomyelitis vaccines. Am J Public Health Nations Health 44(5):563–570
3. Sabin AB (1985) Oral poliovirus vaccine: history of its development and use and current challenge to eliminate poliomyelitis from the world. J Infect Dis 151(3):420–436
4. Furesz J (2006) Developments in the production and quality control of poliovirus vaccines—historical perspectives. Biologicals 34(2):87–90
5. Robbins FC (2004) The history of polio vaccine development. In: Plotkin SA, Orenstein WA (eds) Vaccines. Saunders, Philadelphia, pp 17–30
6. Plotkin SA, Vidor E (2008) Poliovirus vaccine—inactivated. In: Plotkin SA, Orenstein WA, Offit PA (eds) Vaccine. Saunders Elsevier, Philadelphia, pp 605–629
7. van Wezel AL (1967) Growth of cell-strains and primary cells on micro-carriers in homogeneous culture. Nature 216(5110):64–65
8. Ferguson M, Wood DJ, Minor PD (1993) Antigenic structure of poliovirus in inactivated vaccines. J Gen Virol 74(Pt 4):685–690
9. Nathanson N, Langmuir AD (1963) The Cutter incident: poliomyelitis following formaldehyde-inactivated poliovirus vaccination in the United States during the spring of 1955. II. Relationship of poliomyelitis to Cutter vaccine. Am J Epidemiol 78:29.6–60.6
10. European Pharmacopoeia, 2.7.20 (2011) In vivo assay of poliomyelitis vaccine (inactivated). In: European Pharmacopoeia 7.0. pp 226–227
11. Beale AJ, Mason PJ (1962) The measurement of the D-antigen in poliovirus preparations. J Hyg (Lond) 60:113–121
12. Souvras M, Montagnon BJ, Fanget B, Van Wezel AL, Hazendonk AG (1980) Direct enzyme-linked immunosorbent assay (ELISA) for quantification of poliomyelitis virus D-antigen. Dev Biol Stand 46:197–202
13. United States Department of Health Education and Welfare (1968) Biological Products Public Health Service, in 42, Public Health Service (ed). Division of Biological Standards National Institutes of Health, Maryland. pp 32–44
14. Gard S, Wesslen T, Fagraeus A, Svedmyr A, Olin G (1956) The use of guinea pigs in tests for immunogenic capacity of poliomyelitis virus preparations. Arch Gesamte Virusforsch 6(5):401–411
15. Timm EA, Rope EZ, Mc LI Jr (1958) Chick potency tests of poliomyelitis vaccine: basic studies on response. J Immunol 80(5):407–414

16. van Steenis G, van Wezel AL, Sekhuis VM (1981) Potency testing of killed polio vaccine in rats. Dev Biol Stand 47:119–128
17. Wood DJ, Heath AB (1995) A WHO collaborative study of immunogenicity assays of inactivated poliovirus vaccines. Biologicals 23(4):301–311
18. European Pharmacopoeia (2014) In vivo assay of poliomyelitis vaccine (inactivated): test in rats. In: European Pharmacopoeia 8.0. Strasbourg, France
19. Martin J, Crossland G, Wood DJ, Minor PD (2003) Characterization of formaldehyde-inactivated poliovirus preparations made from live-attenuated strains. J Gen Virol 84 (Pt 7):1781–1788
20. Taffs RE, Chernokhvostova YV, Dragunsky EM, Nomura T, Hioki K, Beuvery EC, Fitzgerald EA, Levenbook IS, Asher DM (1997) Inactivated poliovirus vaccine protects transgenic poliovirus receptor mice against type 3 poliovirus challenge. J Infect Dis 175(2):441–444
21. Jason-Moller L, Murphy M, Bruno J (2006) Overview of Biacore systems and their applications. Curr Protoc Protein Sci. Chapter 19:Unit 19 13
22. Pattnaik P (2005) Surface plasmon resonance: applications in understanding receptor-ligand interaction. Appl Biochem Biotechnol 126(2): 79–92
23. Westdijk J, Brugmans D, Martin J, Van't Oever A, Bakker WA, Levels L, Kersten G (2011) Characterization and standardization of Sabin based inactivated polio vaccine: proposal for a new antigen unit for inactivated polio vaccines. Vaccine 29(18):3390–3397
24. Kersten G, Hazendonk T, Beuvery C (1999) Antigenic and immunogenic properties of inactivated polio vaccine made from Sabin strains. Vaccine 17(15–16):2059–2066