Chapter 8

Standardized Methods for Detection of Poliovirus Antibodies

William C. Weldon, M. Steven Oberste, and Mark A. Pallansch

Abstract

Testing for neutralizing antibodies against polioviruses has been an established gold standard for assessing individual protection from disease, population immunity, vaccine efficacy studies, and other vaccine clinical trials. Detecting poliovirus specific IgM and IgA in sera and mucosal specimens has been proposed for evaluating the status of population mucosal immunity. More recently, there has been a renewed interest in using dried blood spot cards as a medium for sample collection to enhance surveillance of poliovirus immunity. Here, we describe the modified poliovirus microneutralization assay, poliovirus capture IgM and IgA ELISA assays, and dried blood spot polio serology procedures for the detection of antibodies against poliovirus serotypes 1, 2, and 3.

Key words Poliovirus, Neutralization, Antibodies, ELISA, IgA, IgM, OPV, IPV, Dried blood spot

1 Introduction

1.1 Poliovirus Microneutralization Assay

The polio microneutralization assay measures neutralizing antibody titers to poliovirus types 1, 2, and 3 using 96-well microtiter plates (it is termed "microneutralization" because the original neutralization assay was performed using larger volumes in culture tubes). The principle of the test is that the anti-poliovirus antibodies in a serum sample will bind to the virus and block infection of susceptible cells. Because poliovirus is cytopathic, virus that is not bound by antibody infects and lyses cells. The amount of neutralizing antibody is quantitated as a titer based on the last serum dilution to protect susceptible cell culture wells from poliovirus infection and cytopathic effect.

The test takes approximately 7 days to complete, from the dilution of sera to staining and reading plates, and data analysis. Each test serum is run in triplicate and diluted from 1:8 to 1:1024; a single 96-well plate contains four test sera (Fig. 1). The three replicates are always tested together in contiguous positions as indicated, and located on the same relative plate number for each

Javier Martín (ed.), *Poliovirus: Methods and Protocols*, Methods in Molecular Biology, vol. 1387,
DOI 10.1007/978-1-4939-3292-4_8,

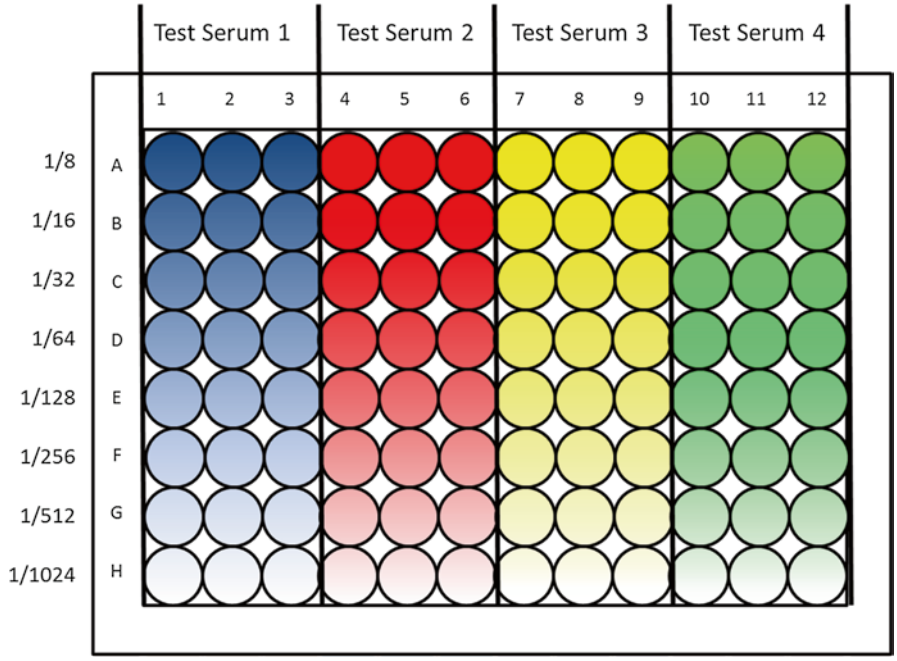

Fig. 1 96-well plate setup for poliovirus microneutralization assay, testing four sera in triplicate against a single virus starting at a 1:8 dilution of sera and twofold dilutions

of the three polio serotypes. This test may be performed manually, automated, or in a combination of the two approaches (Fig. 2). For large studies (>1000 specimens), automation is recommended (*see* **Note 4.4**).

From experience, it was found efficient for work scheduling to design a single run as consisting of up to 92 test sera. Included in each run is a control serum designated In-House Reference Serum (IHRS), which is pooled from serum samples with high neutralizing antibody titers to each Sabin poliovirus. The IHRS is tested in multiple replicates, on multiple plates, in multiple positions in each run (at least four in each run), to provide a measure of assay variability within and between a run. If more than seven sera are being tested, the samples must be randomized using a balanced block randomization scheme. Control plates are generated for each run and consist of three back titration plates with no antibody added (one for each Sabin virus) and a cell control plate (no virus or antibody added, to assess cell viability). At the end of each run, control plates are checked for accurate dilution of each Sabin poliovirus (back titration plates) or cell monolayer confluency (cell control plate).

1.2 Dried Blood Spot Poliovirus Serology

The collection, storage, transport and processing of serum presents a challenge in resource-poor settings and when surveying hard-to-reach or vulnerable populations. Dried blood spots (DBS) are commonly collected for a variety of clinical and public health

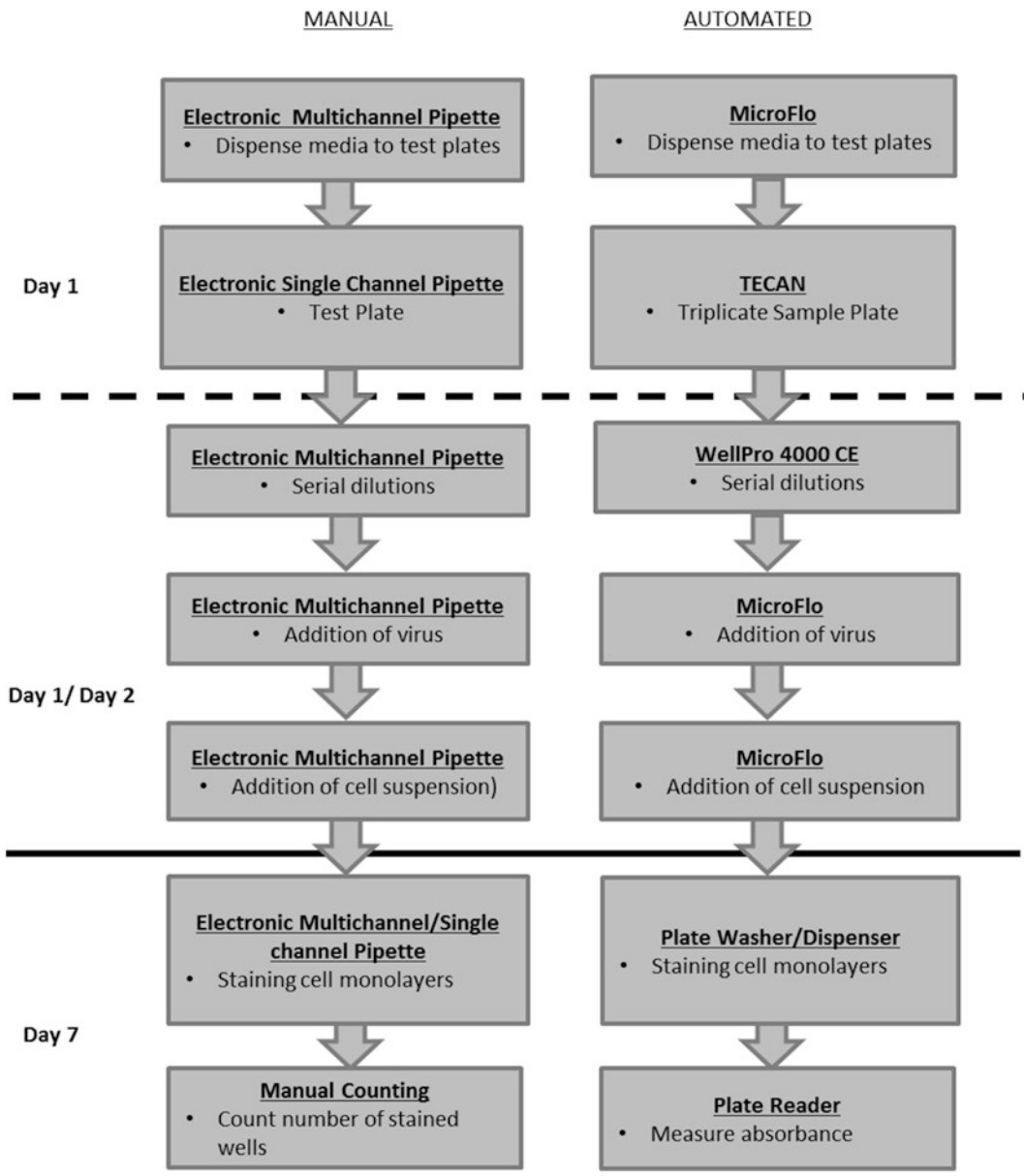

Fig. 2 Workflow for manual and automated poliovirus microneutralization assay

studies, so they are often available for other studies as well. As a result, DBS have been identified as a viable alternative to serum for the detection of a wide range of infections and immune responses. A 6 mm punch from a DBS card contains approximately 6 μl of sera. To achieve a 1:8 serum dilution this assay uses two 6 mm punches to test for polio neutralizing antibodies against Sabin 1, 2, and 3, starting at the standard 1:8 dilution.

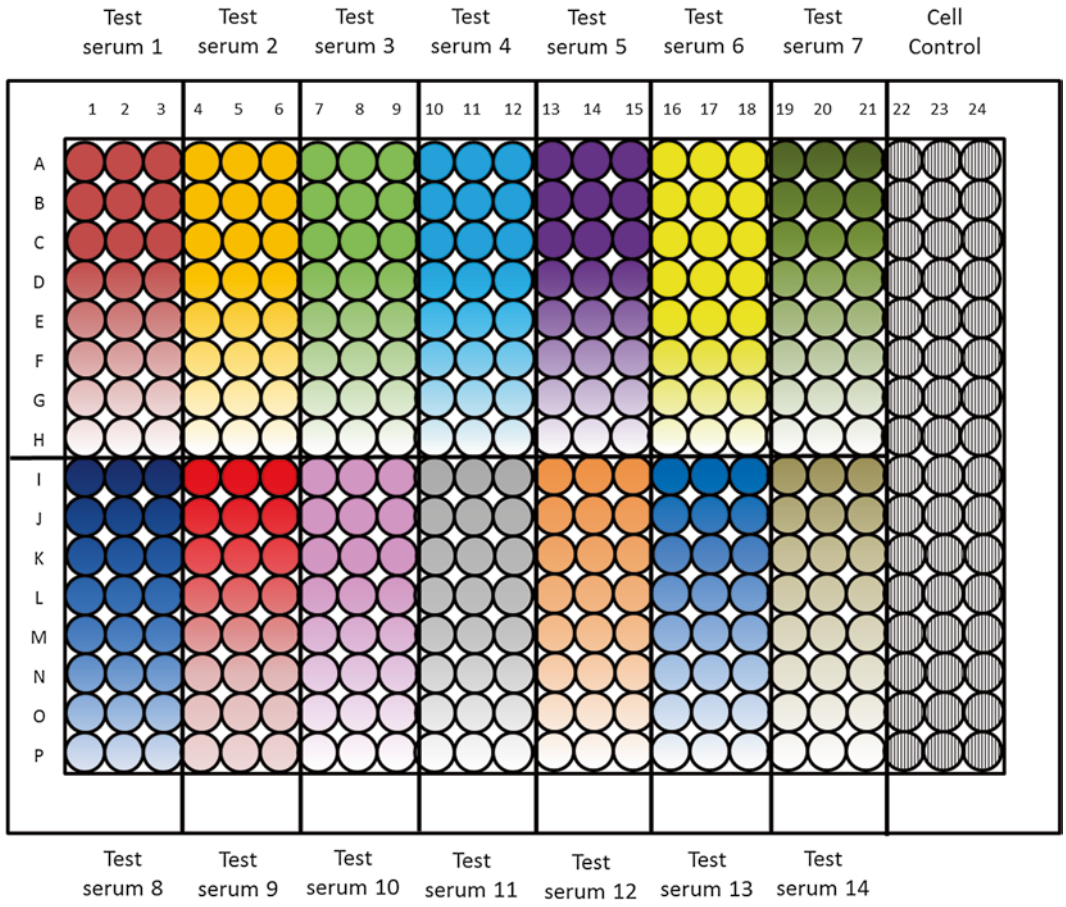

Fig. 3 384-well plate layout for poliovirus dried blood spot microneutralization assay

This procedure is a modification of the SOP for poliovirus serology in 96-well tissue culture plates. The method has been adapted to 384-well plates to allow use of smaller volumes of test sera that are eluted from DBS. Each test serum is run in triplicate and diluted from 1:8 to 1:1024; a single 384-well plate contains 14 test specimens plus cell controls (Fig. 3). The test takes approximately 7 days to complete, from the elution of dried blood spot punches to adding luminescent reagent and reading plates (Fig. 4).

For a single run, up to 252 sera can be tested against each of the three poliovirus serotypes, requiring fifty-four 384-well plates. New control plates are generated for each run and consist of one back titration containing each of three Sabin viruses. At the end of each run, control plates are checked for accurate dilution of each Sabin poliovirus (back titration plate).

If more than 14 sera are being tested, the samples must be randomized using a balanced block randomization scheme with integrated controls (*see* **Note 4.2**). Included in each run is a con-

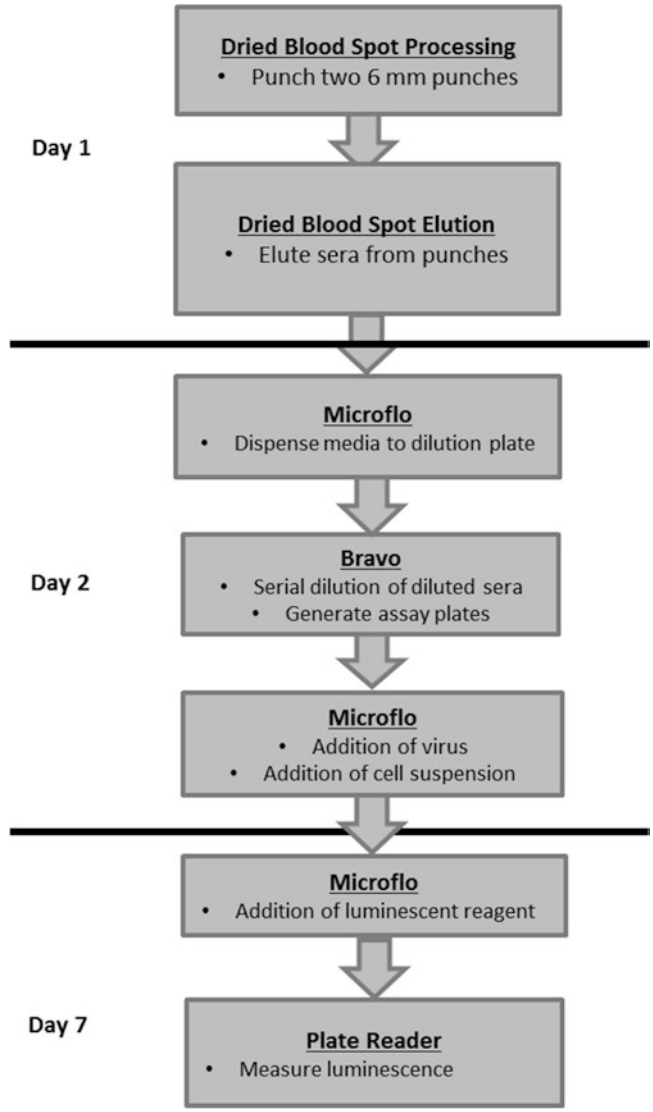

Fig. 4 Workflow for poliovirus dried blood spot microneutralization assay

trol serum designated In-House Reference Serum (IHRS), which is pooled from serum samples with high neutralizing antibody titers to each Sabin poliovirus (*see* **Note 4.1**). Due to the size of the wells and accuracy required for liquid handling on the scale that is necessary for 384-well plates, it is not recommended that this procedure be performed manually.

1.3 Poliovirus IgA/IgM Capture ELISA

The use of the inactivated polio vaccine (IPV) and oral polio vaccine (OPV) has been effective at preventing acute paralytic poliomyelitis and in containing the spread of wild poliovirus almost to the point of eradication. The induction of neutralizing antibody to

each serotype protects against disease and can be detected using a modified microneutralization assay. However, the detection of anti-poliovirus antibody in an ELISA format may represent a more rapid approach to the characterization of a vaccine-induced antibody response and early detection of exposure to live virus, which will be more important in the final stages of eradication and in the immediate post-eradication era.

Immunoglobulin M (IgM) exists as a membrane-bound monomer or a secreted pentamer and is the first antibody produced in response to antigen. Thus, the detection of poliovirus- and enterovirus-specific serum IgM has been proposed as an early detector of exposure to live virus or vaccine. In infants, the presence of antigen-specific IgM in cord blood indicates exposure to the antigen because maternal IgM is unable to cross the placental barrier.

Immunoglobulin A (IgA) is present in both serum and mucosal secretions, such as saliva, stool, and breast milk, as monomers or dimers, and functions to neutralize pathogens and toxins at mucosal surfaces. The mucosal immune response plays a significant role in the response to poliovirus infection and is an important mediator of protection induced by the oral polio vaccine. Poliovirus-specific IgA has been shown to be present in saliva and serum of OPV recipients and individuals exposed to wild poliovirus.

Poliovirus-specific IgM and IgA in biological fluids can be detected with a μ-chain or α-chain capture ELISA or a sandwich ELISA, by using serotype-specific monoclonal antibodies to detect antigen bound by captured IgA or IgM. However, the published capture ELISA protocols use reagents generated in-house and are not easily accessible to other public health and surveillance laboratories worldwide.

2 Materials and Equipment

Most of these items may be substituted with equivalent items from other manufacturers/suppliers but alternative materials must be validated against an appropriate standard. The materials below have been validated for consistent performance in this assay.

2.1 Polio Microneutralization Assay

2.1.1 Consumables and Small Equipment

- T-150 tissue culture flasks (Corning, #430823).
- 96-well tissue culture, clear, sterile plates (Corning, #3997).
- Low evaporation lids (Corning, #3931).
- Plastic wrap (e.g., Saran Wrap).
- Deep-well 96-well microplate, 2.2 ml capacity. (VWR, #40002-014).
- Mat lids for 2.0 ml microplates (VWR, #400002-018).
- Sterile pipettes; 10, 25 ml (Falcon, #357551, #357325).

- Single-channel pipettes:
  - Manual: LTS 20, 200, 1000 (Rainin).
  - Electronic: 10-300, 50-1000 (Biohit).
- 12-channel pipettes.
  - Electronic: 10-300, 50-1200 (Biohit).
- Pipette tips.
  - Rainin: RT-L200F, RT-L1000F, RT-L10F.
  - Biohit: 350, 1000, 1200 µl.

2.1.2 Cells and Media

- HEp-2(C)cells (ATCC # CCL23).
- MEM + 10 % fetal bovine serum (FBS) (*see* **Note 4.6**).
- MEM + 2 % FBS (*see* **Note 4.6**).

2.1.3 Antigens and Control Sera

- In-House Reference Sera (developed in-house) (*see* **Note 4.1**).
- Sabin virus stocks grown in HEp-2(C)cells (*see* **Note 4.3**).

2.1.4 Staining

- Crystal violet stain (0.05 % crystal violet, 0.5 % Tween 20, 24 % ethanol, in H_2O).

To prepare crystal violet stock solution:	
2 g	Crystal violet (Sigma, C-3386)
1000 ml	95 % ethanol

Mix together overnight, with stirring, until dissolved; may be stored up to 1 year at room temperature

To prepare working dilution crystal violet solution:	
250 ml	Crystal violet stock solution
5 ml	Tween 20 (Fisher Scientific, #BP337-100)
745 ml	Deionized H_2O

2.1.5 Other Equipment

- CO_2 water-jacketed incubator (Thermo Fisher, Model 3110 or equivalent).

2.2 Dried Blood Spot Poliovirus Serology

2.2.1 Consumables and Small Equipment

- 150 cm^2 tissue culture flasks (Corning, #430823).
- 384-well, white, opaque cell culture plates w/lids (PerkinElmer, #3596).
- 384-well, v-bottom plates (Matrix, Technologies; Fisher Scientific, Cat no. 50-823-850).
- Deep-well 96-well microplate, 0.5 ml capacity (VWR, #40002-022).
- 96-well, filter-bottom plates (PALL, Cat no. 8079).

- MicroClime Environmental lid (Labcyte, Cat no. LL-0301-IP).
- Plastic wrap (e.g., Saran Wrap).
- Sterile pipettes; 10, 25 ml (Falcon, #357551, #357325).
- Single-channel pipettes:
  - Manual: LTS 20, 200, 1000 (Rainin).
  - Electronic: 10-300, 50-1000 (Biohit).
- 12-channel pipettes.
  - Electronic: 10-300, 50-1200 (Biohit).
- Pipette tips.
  - Rainin: RT-L200F, RT-L1000F, RT-L10F.
  - Biohit: 350, 1000, 1200 µl.
  - Viaflow: 125 µl, 384 tips; filtered, sterile (#4425).

2.2.2 Cells and Media

- HEp-2(c)cells (ATCC # CCL23).
- MEM + 10 % FBS (*see* **Note 4.6**).
- MEM + 2 % FBS (*see* **Note 4.6**).

2.2.3 Antigens and Control Sera

- In House Reference Sera (generated in-house).
- Sabin virus stocks grown in HEp-2(c) cells.

2.2.4 Other Equipment

- CO_2 water-jacketed incubator (Thermo Fisher, Model 3110 or equivalent).
- ATPlite (PerkinElmer).
- Eppendorf refrigerated centrifuge (Model 5810R or equivalent with rotor capable of accommodating 96-well plate).
- MicroFlo (BioTek), or equivalent.
- BioStack2 (microplate Stacker) (BioTek), or equivalent.
- Automatic cell counter (Bio-Rad, TC-20), or equivalent.
- Victor X4 Multimode Reader (PerkinElmer), or equivalent.
- Bravo Liquid Handling System (Agilent) with 384-channel disposable tip manifold, or equivalent.
- Wallac DBS Puncher (PerkinElmer), or equivalent or manual puncher.
- 8-channel spanning-head pipette, Viaflow Voyager (Integra), or equivalent.

2.3 Poliovirus IgA/IgM Capture ELISA

2.3.1 Antibodies

1. **Capture antibodies:**
   - Affinity-purified antibody, anti-human IgA (α) (Kirkegaard & Perry Laboratories, Inc.; catalog no. 01-10-01).
   - Affinity-purified antibody, anti-human IgM (µ) (Kirkegaard & Perry Laboratories, Inc.; catalog no. 01-10-03).

2. **Monoclonal antibodies:**

Each monoclonal antibody was screened for specificity to their respective antigen by direct ELISA.

- Anti-poliovirus type-1 IPV (Antibody Shop ; HYB 295-17-02).
- Anti-poliovirus type-2 IPV (Antibody Shop ; HYB 294-06).
- Anti-poliovirus type-3, IPV (Antibody Shop ; HYB 300-06).

Antibody Shop monoclonal antibodies are supplied in 200 µl volumes. To store the monoclonal antibodies properly, add 200 µl of glycerol and store the monoclonal antibodies at –20 °C.

- Anti-poliovirus type-1, Sabin (Millipore ; MAB8560).
- Anti-poliovirus type-2, Sabin, (Millipore ; MAB8562).
- Anti-poliovirus type-3, Sabin, (Millipore ; MAB8564).

3. **Detector antibody:**

- Goat anti-mouse IgG (H+L), human-serum-adsorbed, horseradish-peroxidase-labeled, (Kirkegaard & Perry Laboratories, Inc., catalog no. 074-1806).

2.3.2 Equipment and Supplies

- Immulon 2HB, 96-well polystyrene, flat-bottom, high-binding. Thermo no. 3455 (Thermo Fisher cat. no. 14-245-61).
- Class II biosafety cabinet (BSC).
- Incubator (Precision) (37 °C).
- Multichannel Pipette (Gilson 50–200 µl).
- Pipettes: (Gilson LTS 2, 10, 20, 200, 1000 µl).
- Deep well dilution racks (VWR).
- 15 and 50 ml centrifuge tubes (Falcon).
- 2, 5, 10, and 25 ml serological pipettes (Falcon).
- Reagent reservoirs (Costar).
- Balance (Ohaus, Adventurer).
- Stir plate (Thermolyne).
- ELISA washer (BioTek), inside BSC.
- ELISA plate reader (Victor X4, or equivalent).

2.3.3 Buffers

- **Carbonate buffer:** 0.05 M, pH 9.6, plus 0.02 % sodium azide.
- **Wash buffer:** PBS: phosphate-buffered saline (0.01 M) pH 7.2 with Tween 20 (0.05 %).
- **Blocking and dilution buffer:** P-G-T, PBS (0.01 M) pH 7.2, 0.5 % gelatin, 0.15 % Tween 20: PBS (0.01 M, pH 7.2), Difco Gelatin, Fisher cat. no. DF0143-17-9, polyoxyethylene-sorbitan monolaurate (Tween 20), Sigma cat. no. P-1379.

2.3.4 Antigens

- IPV1: RIVM inactivated poliovirus type-1, Pu96-1285-907.
- IPV2: RIVM inactivated poliovirus type-2, Pu97-273-907.
- IPV3: RIVM inactivated poliovirus type-3, Pu05-3454-907.
- Sabin 1: NIBSC 01/528, RD2, 2/10/2009. (5.1 log $CCID_{50}$).
- Sabin 2: NIBSC 01/530, RD2, 2/10/2009. (5.1 log $CCID_{50}$).
- Sabin 3: NIBSC 01/532, RD2, 2/10/2009. (5.3 log $CCID_{50}$).

3 Protocol

3.1 Polio Microneutralization Assay

3.1.1 In the Week Preceding the Test Runs

1. Assign sera randomly to each run using a balanced block randomization scheme (*see* **Note 4.2**).
2. Use the list generated by the randomization scheme to label plates and organize sera to be tested. Each test serum is run in triplicate, so four sera may be run on each plate (Fig. 1). Each plate is duplicated two more times, yielding three plates, one for each poliovirus serotype. There will also be a back-titration plate for each serotype, and one cell control plate per run. In a typical 96 sera run, there are 76 plates numbered as follows: PV1 virus back titration: 1; PV2 virus back titration: 2; PV3 virus back titration: 3; Cell control: 4; Sera against PV1: 5–28; Sera against PV2: 29–52; and Sera against PV3: 53–76.
3. *See* Table 1 for an example of a randomized sample list. In this example, serum sample number 0000000009 will be in run number 420, position 4 on plates 5 (PV1), 28 (PV2), and 53 (PV3). The in-house reference serum (IHRS) is in position 2 on plates 7 (PV1), 30 (PV2), and 55 (PV3).
4. For each run, the IHRS is tested an average of 4–6 times, depending on the number of samples being randomized. The IHRS sera are randomized with the test sera and are not in the same plate or position for every run.
5. Prepare MEM+ 2 % FBS and MEM + 10 % FBS according to **Note 4.6**.

3.1.2 Prior to Each Test Run

1. 24–48 h before each run, seed T-150 flasks with 30 ml of HEp-2(C) cells at 5×10^5 cells/ml.
   (a) Approximately 3–4 flasks are needed for one run of 96 sera.
2. Incubate flasks at 37 °C, 5 % CO_2, in a humidified atmosphere for 24–48 h to ensure that cell monolayers are confluent the day assay runs are started.

3.1.3 Optional: 1 Day before Test Run

The following steps can be done the day of the run or the day before the addition of the virus. Steps 6 and 7 can be performed using automation (see Subheading "Data Collection" in Note 4.4)

Table 1
Example of randomized sample list for poliovirus microneutralization assay

RUN[a]	PV1 Plate[b]	PV2 Plate[b]	PV3 Plate[b]	Position[c]	Sample ID[d]
420	5	28	53	1	0000000001
420	5	28	53	2	0000000002
420	5	28	53	3	0000000006
420	5	28	53	4	0000000009
420	6	29	54	1	0000000011
420	6	29	54	2	0000000030
420	6	29	54	3	0000000025
420	6	29	54	4	0000000041
420	7	30	55	1	0000000013
420	7	30	55	2	IHRS
420	7	30	55	3	0000000038
420	7	30	55	4	0000000051

[a]Each run of 1–96 sera
[b]PV1, polio type 1; PV2, polio type 2; PV3, polio type 3
[c]*See* Fig. 1
[d]Unique specimen ID and IHRS (in-house reference serum)

1. Manually aliquot 100 µl of each serum sample into deep-well polypropylene microplate, sealed with a mat lid to prevent contaminations, and heat-inactivated at 56 °C in a water bath for 30 min.
2. Store at 4 °C until ready to transfer samples to assay plates (no more than 24 h).
3. Prepare IHRS for testing (*see* **Note 4.1**).
4. Use a multichannel pipette to add 300 µl MEM + 2 % FBS to 100 µl heat-inactivated test serum aliquots (for a final dilution of 1:4)
5. For a full run (i.e., 96 sera), label two stacks of 12 microplates for each serotype (Fig. 5).
   (a) Use a lidded microplate for the top of each stack, with the low evaporation lid; the 11 remaining microplates should be lidless.
   (b) Cell control and back titration plates can be set up in lidded plates.

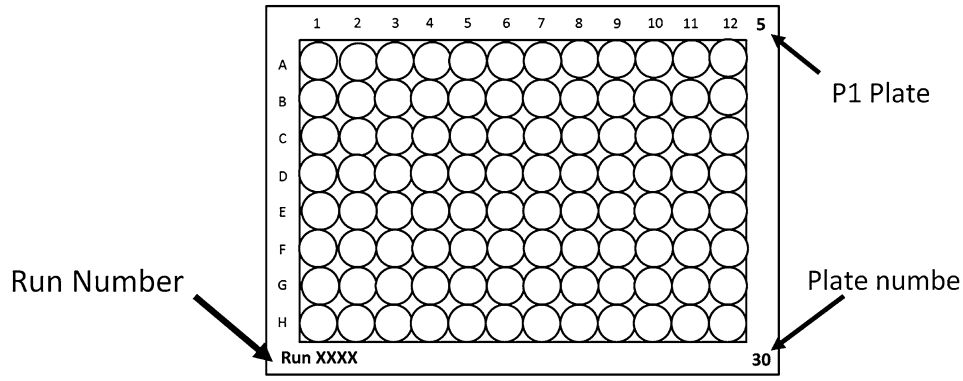

Fig. 5 Suggested labeling scheme for assay plates. Each plate should indicate the run number in the *bottom left* corner, the plate number in the *bottom right* corner, and the P1 plate number in the *upper left* corner as a reference point

6. Use an electronic multichannel pipette to dispense 25 µl MEM + 2 % FBS to the test plates.
   (a) Add 25 µl MEM + 2 % FBS to each well of the back titration plates (PV1, PV2, and PV3).
   (b) Add 50 µl MEM + 2 % FBS to each well of the cell control plate.
7. Use an electronic single-channel pipette to transfer 25 µl of each test serum (from **step 4**) to each test plate in triplicate (Fig. 6a).
8. Cover the top plate of each stack of plates and wrap in plastic wrap.
9. Plates can be stored overnight at 4 °C.

3.1.4 Day of Run

Steps 1, 4, and 9 can be performed using automation (see Subheading "Day of Run" in Note 4.4). These steps should be performed under a biological safety cabinet.

1. Using a multichannel pipette, make serial twofold dilutions from row A to row H. (serum dilution will range from 1:8 to 1:1024) (Fig. 7).
   (a) Discard 25 µl from row H to make final volume for all wells 25 µl.
2. Dilute each virus in MEM + 2 % FBS to contain 100 $CCID_{50}$/25 µl.
   (a) *See* Table 2 for example dilution scheme.
   (b) Prepare sufficient virus challenge suspension for the number of sera to be tested; each plate requires approximately 2.5 ml of diluted challenge virus.

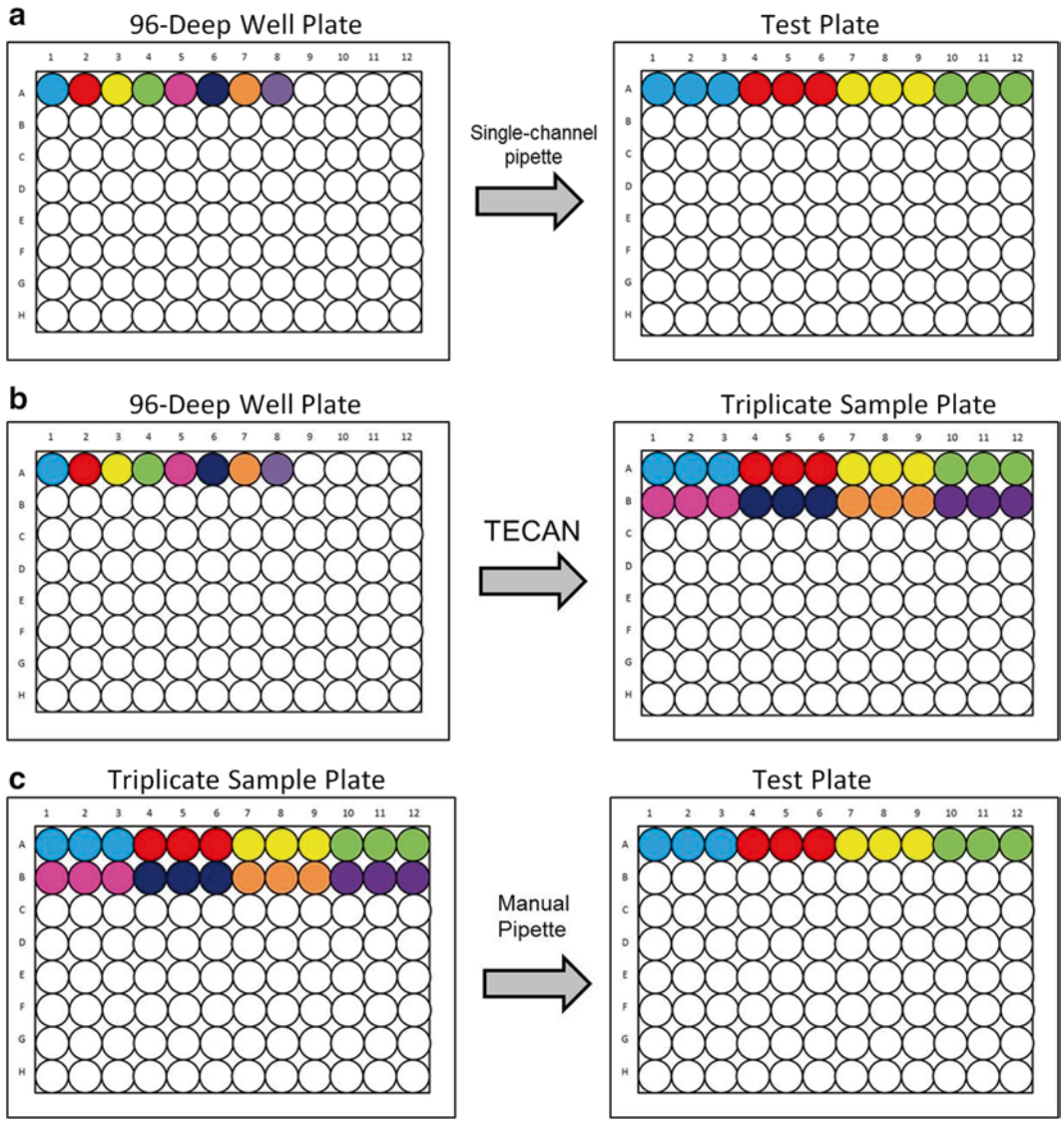

Fig. 6 For each run, 100 μl of sample is transferred from sample plate to dilution plates in triplicate (enough for starting three 25 μl sample dilutions). For simplicity, only the first eight samples are shown in the deep 96-well plate. (a) Using a single-channel pipette, transfer each sample from the 96-deep well plate to a test plate in triplicate. (b) The TECAN configuration will allow for triplicates in consecutive order. This step can transfer 96 samples from one 96-deep well plate to a 96-well triplicate sample plate in 22 min. (c) Using a 12-channel manual pipette, each row of from the triplicate sample plate is transferred to a test plate in triplicate for each serotype

3. Prepare the back titrations of each poliovirus serotype in MEM + 2 % FBS. Titrate each virus from 100 $CCID_{50}$ a further 3 tenfold steps (Table 2).
4. Use an electronic, multichannel pipette to add 25 µl of 100 $CCID_{50}$ of relevant poliovirus antigen to all wells in the diluted test plates.

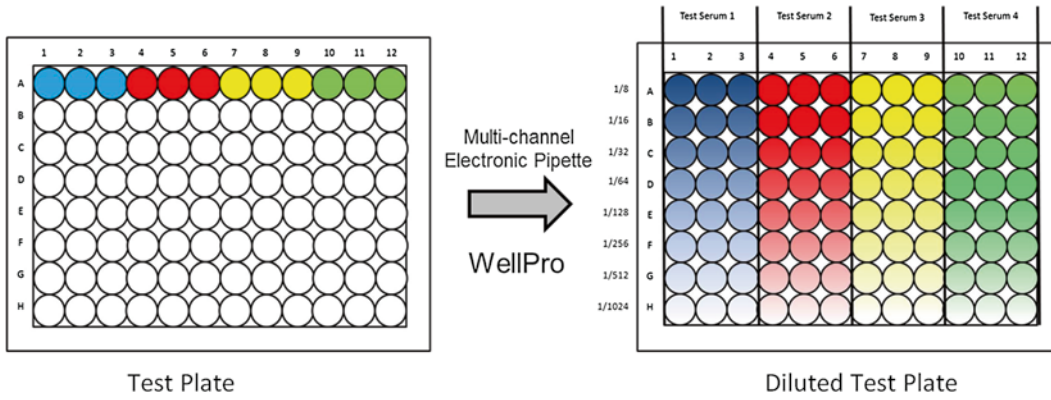

Fig. 7 Each test serum is serially diluted (twofold) from 1:8 to 1:1024 in triplicate. This is repeated for each test plate designated for poliovirus serotypes 1, 2, or 3 and can be done using a multichannel electronic pipette or automation

Table 2
Example virus dilution for 100 $TCID_{50}$ and back titration plate for Sabin type 1, 2, and 3

Sabin 1 (NIBSC 01/528)						**100 $TCID_{50}$**	**10 $TCID_{50}$**	**1 $TCID_{50}$**	**0.1 $TCID_{50}$**
100 $TCID_{50} = 10^{-5.28}$									
Virus	100 µl	100 µl	100 µl	100 µl	800 µl	7 ml	100 µl	100 µl	100 µl
Medium	38 µl	900 µl	900 µl	900 µl	7.2 ml	63 ml	900 µl	900 µl	900 µl
Sabin 2 (NIBSC 01/530)									
100 $TCID_{50} = 10^{-5.20}$									
Virus	100 µl	100 µl	100 µl	100 µl	800 µl	7 ml	100 µl	100 µl	100 µl
Medium	82 µl	900 µl	900 µl	900 µl	7.2 ml	63 ml	900 µl	900 µl	900 µl
Sabin 3 (NIBSC 01/532)									
100 $TCID_{50} = 10^{-4.68}$									
Virus	100 µl	100 µl	100 µl		800 µl	7 ml	100 µl	100 µl	100 µl
Medium	82 µl	900 µl	900 µl		7.2 ml	63 ml	900 µl	900 µl	900 µl
						Working stock	Back titration plate		

5. Prepare back titration plate (Fig. 8) for each Sabin strain using dilutions prepared in **step 2** (Table 2).
   (a) Add 25 µl of 100 $TCID_{50}$ of virus to rows A and B (i.e., 24 wells/dilution)
   (b) Add 25 µl of the next 3 tenfold dilutions to rows C and D, E and F, and G and H, respectively (*see* Table 2 for dilutions).
   (c) ***Change pipette tips between each dilution.***

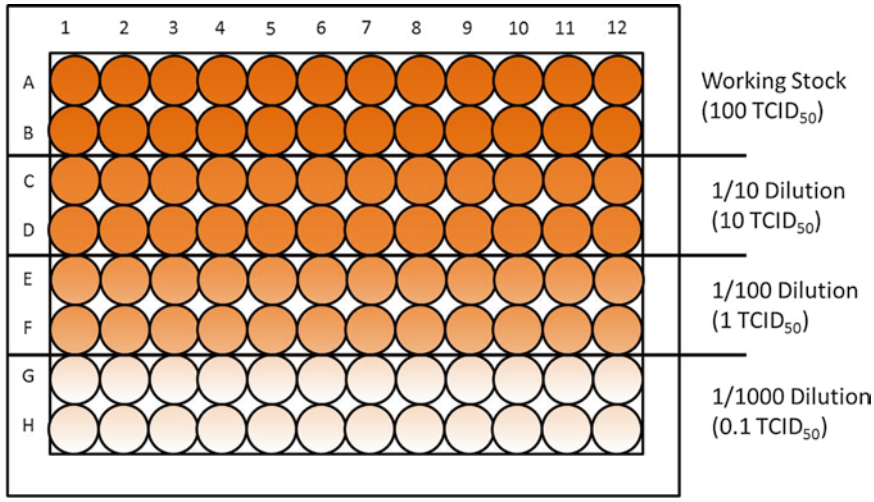

Fig. 8 Layout for back titration plate for the poliovirus microneutralization assay. A back titration plate is made for each virus tested in the assay

6. Wrap all plates in plastic wrap and incubate for 3 h at 35 °C and 5 % CO_2.
7. During serum-virus incubation, wash HEp-2(C) monolayer cell cultures (from 150 cm^2 flasks), trypsinize, and count cells using automatic cell counter (Bio-Rad) or a hemocytometer.
8. Prepare a HEp-2(C) cell suspension in MEM + 10 % FBS to contain 3×10^5 cells/ml. Prepare a sufficient volume of cells: each plate requires approximately 2.5 ml of cell suspension, and every run requires 3–4 confluent 150 cm^2 flasks. Store cells in glass bottle at 4 °C until ready to use.
9. Use a repeating, multichannel pipette to add 25 μl of prepared cell suspension to each well of every plate.
10. Wrap all plates in plastic wrap, in stacks of 12–13 plates. To prevent spills and cross-contamination, ***avoid abrupt handling of plates.***
11. Carefully transfer plates to incubator for 5 days incubation at 35 °C and 5 % CO_2.

3.1.5 Plate Washing and Staining

*The steps in Subheading 3.1.5 can be performed using automation (see Subheading "**Plate Washing and Staining**" in Note 4.4). These steps should be performed in a biological safety cabinet.*

1. After 5 days incubation, aspirate/discard media with multichannel pipette or a vacuum into freshly made 0.5 % sodium hypochlorite solution.

2. Using a repeating, multichannel pipette, add 50 μl crystal violet stain (0.05 %) to all plates.
3. Incubate for a minimum of 40 min at room temperature.
4. Aspirate/discard stain with multichannel pipette, fill each well with tap water (approximately 250–300 μl), and discard.
5. Repeat washing **step 3** more times.
6. Allow plates to dry at room temperature for at least 2 h under a biological safety cabinet.
7. Once dried, plates can be stored outside of a biological safety cabinet at room temperature until results are calculated and reported.

3.1.6 Data Collection

*The steps in Subheading 3.1.6 can be performed using automation (see Subheading "**Data Collection**" in Note 4.4).*

1. For each triplicate test serum, count the total number of wells positive for neutralization (i.e., purple wells).
2. To calculate a neutralization titer:

$$\text{Titer} = \left(\#\text{positive wells} / \#\text{replicates}\right) + 2.5$$

3. To calculate reciprocal titer:

$$\text{Reciprocal titer} = 1:2^{\text{titer}}$$

4. For the neutralization titers, the upper limit of detection is 10.5 and the lower limit is 2.5, which is considered negative.

3.1.7 Cross-Checking Stained Plates

To insure accuracy, each plate is cross checked to verify correct order of plates and compare plate staining pattern to electronic data file to verify titer (Fig. 9).

1. Due to biological and technical (i.e., pipetting errors) variations in the assay, there is a likelihood that some wells will have virus not neutralized by antibody despite neutralization at lower serum dilutions.
2. Within one dilution of the endpoint this can be stochastic because of small amounts of antibody. Further from the endpoint it can be either obvious or not related to technical problems and/or errors. Another possibility is that virus is omitted from a well or row of wells and then incorrectly appear as neutralization.
3. To account for this, stained plates should be checked for these situations and the data adjusted. In the examples below, refer to Fig. 9.

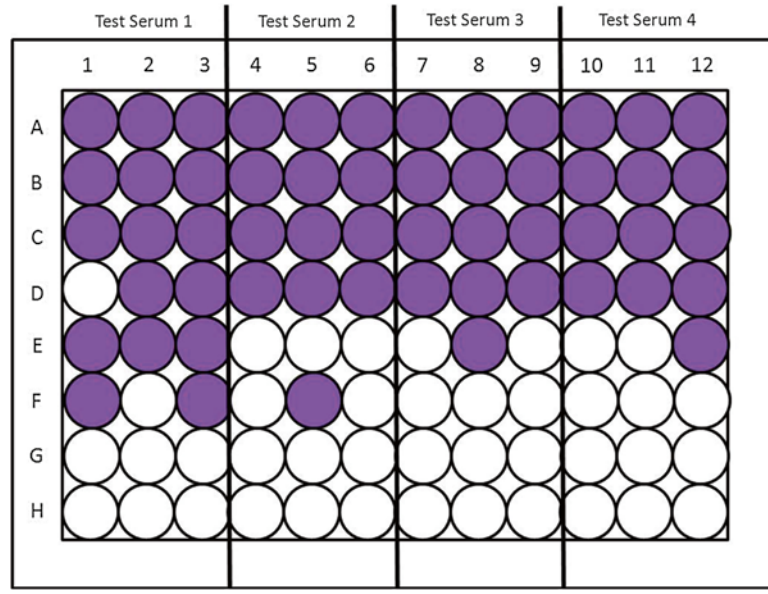

Fig. 9 Example of poliovirus microneutralization assay plate stained with crystal violet for cross-checking. Due to variations in the assay (biological and technical), there is a likelihood that the stained plates will have some wells negative for neutralization despite neutralization at lower serum dilutions

Example 1

(a) For Test Serum 1, the reader software will automatically read this as a titer of 7.83 (1:227).

(b) By cross-checking the plate, we will count D1 as positive for neutralization because the 1:128 and 1:256 dilutions (E1 and F1) are positive for neutralization (i.e., purple).

(c) Using Formula 1, the titer is adjusted to 8.17 (1:288).

Example 2

(a) For Test Serum 2, the reader software will automatically read this as a titer of 6.83 (1:114).

(b) By cross-checking the plate, we will count F5 as negative for neutralization because the 1:64 dilutions (D4, D5, and D6) are negative for viral growth.

3.1.8 Quality Control Analysis for Polio Microneutralization Assay

In-House Reference Serum

1. As the data is collected for each run, the neutralization titers for the in-house reference serum should be collected and summarized for quality control analysis.
2. The median IHRS neutralization titer for each run should be within a ±1.0 $\log_2$ range from the established titer for a stock of IHRS.
3. Within a run, the standard deviation for the IHRS neutralization titer should not exceed ±0.5 $\log_2$.

4. If there is any deviation from the ranges indicated above, the microneutralization run should be repeated. These IHRS titers should be monitored over time.

Back Titration Plates

1. As the data is collected for each run, the data for the back titration plates for each poliovirus tested should be collected and summarized for quality control analysis.
2. Calculate the titer for each poliovirus using the following formula:

$$Log\text{CCID}_{50} = S - 0.5, \text{ where}$$

S = sum of proportion of positive wells.

3. The expected titer calculated from each back titration plate is 2.00 ($\log_{10}$), corresponding to 100 $CCID_{50}$.
4. For each run, the titer for each poliovirus tested should be between 1.5 and 2.5 corresponding to 32 and 320 $CCID_{50}$, respectively.
5. If there is any deviation from this range, the $CCID_{50}$ for the virus stocks should be repeated, the dilution for the microneutralization assay recalculated, and the microneutralization run should be repeated. These virus titers should also be monitored over time.

3.2 Dried Blood Spot Poliovirus Serology

3.2.1 In the Week Preceding the Test Runs

1. Assign sera randomly to each run using a balanced block randomization scheme (*see* **Note 4.2**).
2. Each test serum is run in triplicate, so 14 sera may be tested on each plate (Fig. 3). Each plate is duplicated two more times for the other two poliovirus serotypes. For each run, a single back-titration plate is required to monitor the quality for each run.
3. In Table 3, serum sample number 0000000009 will be in run number 420, position 4 on plates 2 (PV1), 20 (PV2), and 38 (PV3). The in house reference serum (IHRS) is in position 10 on plates 2 (PV1), 20 (PV2), and 38 (PV3).
4. For each run, the IHRS is tested 5–10 times, depending on the number of samples being randomized.
5. Prepare MEM+ 2 % FBS and MEM + 10 % FBS according to **Note 4.6**.

3.2.2 Dried Blood Spot Punch Collection and Elution

1. Pretreat 96-well filter-bottom plate with 200 µl MEM + 2 % FBS to wet filter.
2. Centrifuge at 1500 × *g* for 10 min. Discard flow-through.
3. Using PerkinElmer DBS Processor or manual punch, remove three 6 mm punch from dried blood spots into 96-well filter bottom plate (Fig. 10).
4. Add 68 µl MEM +2 % FBS to all wells of filter plate.

Table 3
Example of randomized sample list for poliovirus microneutralization assay

RUN[a]	PV1 Plate[b]	PV2 Plate[b]	PV3 Plate[b]	Position[c]	Study ID[d]
420	2	20	38	1	0000000001
420	2	20	38	2	0000000002
420	2	20	38	3	0000000006
420	2	20	38	4	0000000009
420	2	20	38	5	0000000011
420	2	20	38	6	0000000030
420	2	20	38	7	0000000025
420	2	20	38	8	0000000041
420	2	20	38	9	0000000013
420	2	20	38	10	IHRS
420	2	20	38	11	0000000038
420	2	20	38	12	0000000051
420	2	20	38	13	0000000098
420	2	20	38	14	0000000120
420	3	21	39	1	0000000087
420	3	21	39	2	0000000067
420	3	21	39	3	0000000199

[a]Each run of 1–270 sera
[b]PV1,polio type 1; PV2, polio type 2; PV3, polio type 3
cSee Fig. 1
[d]Unique specimen ID

5. For wells designated for IHRS, add 12.5 µl of diluted IHRS following guidelines for preparation of the IHRS (*see* **Note 4.1**).
6. Replace plate cover and wrap in plastic wrap.
7. Elute DBS punches by incubation at 4 °C overnight.
8. Remove DBS filter plate from refrigerator.
9. Set elution plate on 96-deep well 0.5 ml plate. Centrifuge at 1500 × *g* for 10 min.
10. Replace lid on V-bottom plate and discard filter plate.
    (a) DBS elution plate contains 50 µl of 1:4 dilution of each serum from DBS punch.
11. Store at 4 °C until ready to begin serology run (not to exceed 24 h).

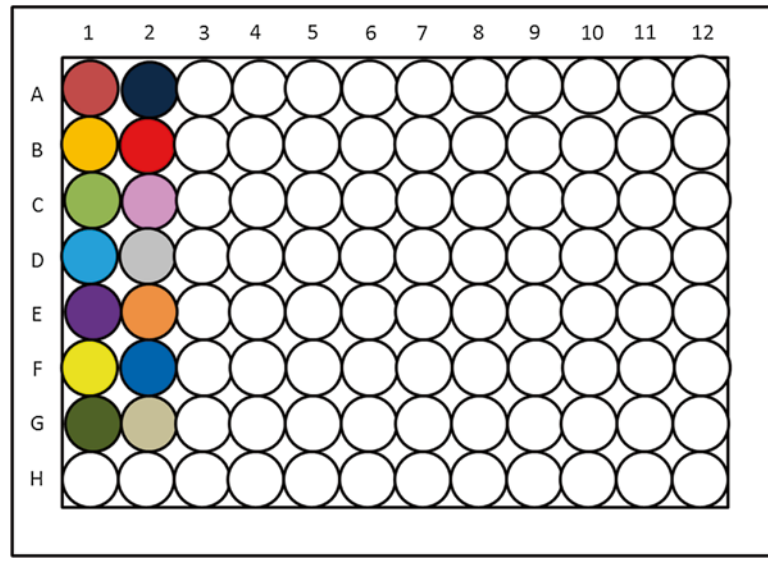

Fig. 10 Filter plate layout for dried blood spot serum elution. For simplicity, only the first 14 specimens are shown

3.2.3 Day of Serology Run

1. Use the Microflo to add 30 µl MEM + 2 % FBS to all rows of a 384-well V-bottom plate.
2. Using the Viaflow Voyager, transfer 30 µl of the first seven diluted sera to row A of dilution plate in triplicate (Fig. 11).
   (a) Repeat for the second set of seven sera to row I.
   (b) This will generate the 1:8 dilution.
3. Use the Bravo to perform twofold serial dilutions in rows A-H and rows I-P to generate a dilution plate (Fig. 12). For a single run, there will be 18 dilution plates.
4. Use the Bravo to transfer 3 µl of each well from a dilution plate to three assay plates.
   (a) Each assay plate corresponds to the serotype being tested.
5. Dilute each poliovirus serotype in MEM + 2 % FBS to contain 100 $CCID_{50}$/3 µl.
   (a) Prepare sufficient virus challenge suspension for the number of sera to be tested. A single plate requires approximately 1.5 ml of diluted challenge virus.
   (b) It is recommended to prepare an additional 1.5 ml of virus to account for priming the Microflo prior to diespensing.
6. Prepare the back titrations of each poliovirus serotype in MEM + % FBS. Dilute each virus from 100 $CCID_{50}$ a further 3 tenfold steps. These dilutions will be used in **step 9**.
7. Use the MicroFlo to add 3 µl of 100 $TCID_{50}$ of relevant poliovirus to all wells in the test plates. Use a different sterile dispensing cartridge for each virus.

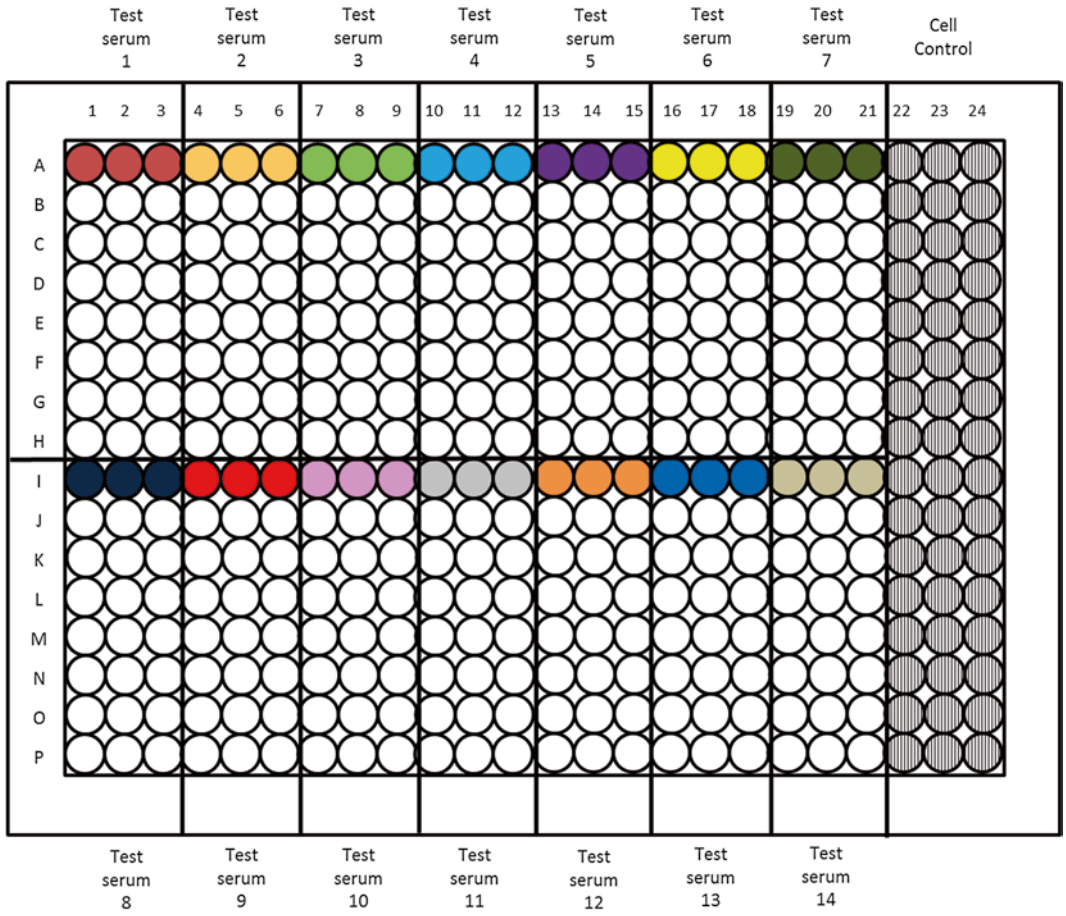

Fig. 11 Dilution Plate layout generated from sample plate using the Viaflow Voyager

(a) Quick spin all plates on a table top plate centrifuge.

8. Prepare back titration plate (Fig. 13) for each Sabin strain using dilutions prepared in **step 5**.
9. Use the Microflo to add 3 µl of MEM + 2 % FBS to all wells of a 384-well flat bottom white opaque plate.
   (a) Add 3 µl of 100 $CCID_{50}$ of Sabin 1, 2 , and 3 to rows A, F, and K (i.e., 24 wells/dilution).
   (b) Add 3 µl of the next 3 tenfold dilutions to rows B, C, and D for Sabin 1; G, H, I for Sabin 2; L, M, and N for Sabin 3.
   (c) Rows E, J, O, and P are reserved for cell only controls.
   (d) Add an additional 3 µl of MEM + 2 % FBS to cell control wells.
10. Replace the top plate with a MicroClime environmental lid that has been moistened with sterile water.
11. Wrap all plates in plastic wrap (ten plates per stack) and incubate for 3 h at 35 °C and 5 % CO_2.

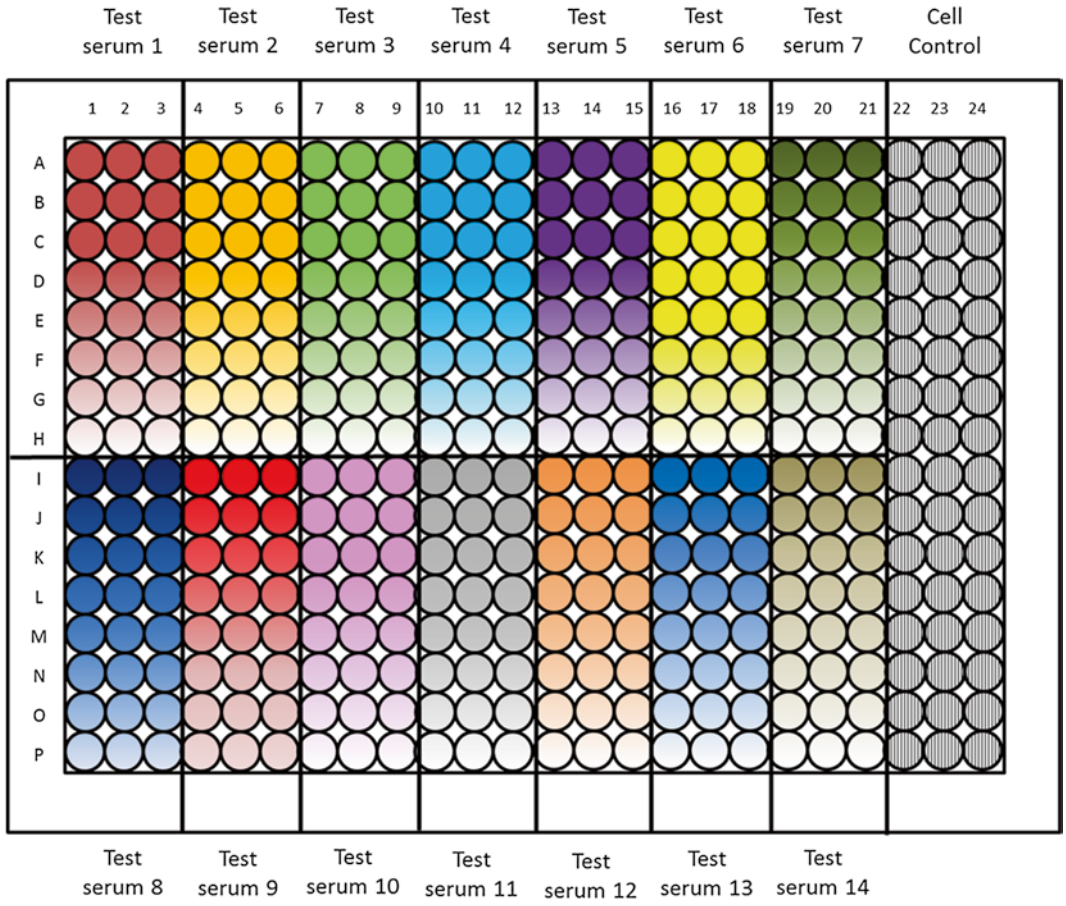

Fig. 12 Dilution Plate after Bravo performs twofold serial dilution

12. During antigen-serum incubation, prepare HEp-2(c) cell suspension in MEM + 10 % FBS to contain 7.5×10^4 cells/ml.
    (a) Prepare a sufficient volume of cells; each plate requires approximately 9 ml of cell suspension, and every 20 plates require one to two 150 cm^2 flasks of confluent cells.
    (b) Store cells in glass bottle at 4 °C until ready to use.
13. Use the MicroFlo to add 20 µl of prepared cell suspension to each well of every plate (Fig. 5).
14. Wrap all plates in plastic wrap and tap gently to mix. Incubate for 5 days at 35 °C and 5 % CO_2.

3.2.4 Detection of Viral CPE

1. After 5 days incubation, remove ATPlite Kit from refrigerator and allow Mammalian Cell lysis buffer, substrate, and substrate buffer to reach room temperature.
2. Use the Microflo to add 13 µl of Mammalian Cell Lysis buffer to all plates for each serotype.
3. Incubate for at least 10 min at room temperature.
4. For the Sabin 1 test plates (18 plates for one run), use the Microflo to add 13 µl of Substrate solution.

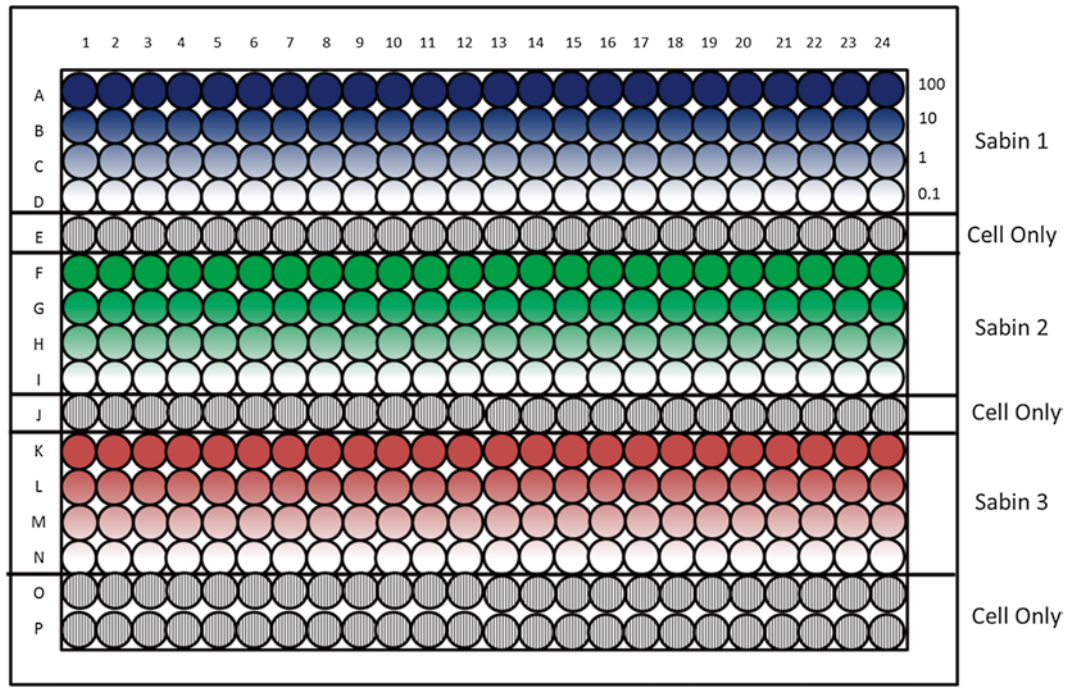

Fig. 13 Back titration plate layout for the poliovirus dried blood spot microneutralization assay

5. Incubate for 10 min at room temperature.
6. Read plates with Victor X4 Multimode plate reader using the luminescent function.
7. As the Sabin 1 plates are read, repeat **steps 5** and **6** for Sabin 2 and 3.

3.2.5 Data Collection

1. For each plate, a cell control is included in columns 22–24 for cutoff calculations.
2. The cutoff for positive/negative wells for neutralization is determined by calculating 80 % of the average luminescence signal for the cell control wells on each plate.
   (a) Wells on test plate below cutoff are considered negative for neutralization (assigned a value of 0).
   (b) Wells on test plate above the cutoff are considered positive for neutralization (assigned a value of 1).
3. Titers can be calculated in Excel using the following formulas:

Formula 1

$$\text{Titer} = \left(\#\text{positive wells} / \#\text{replicates}\right) + 2.5$$

(a) To calculate reciprocal titer:

Formula 2

$$\text{Reciprocal titer} = 1:2^{\text{titer}}$$

3.3 Polio IgA/IgM Capture ELISA

Always check stocks of capture and detector antibodies. The following should be performed the day before microplates are coated with the capture antibodies if no stock is available at –20 °C.

3.3.1 Preparation of Capture and Detector Antibodies

1. Add 500 µl of sterile water to the lyophilized pellet for the capture and/or detector antibodies and rotate until dissolved.
2. Add 500 µl of glycerol and thoroughly mix by pipetting.
3. Allow to reconstitute at –20 °C for 18–24 h.

3.3.2 Coating Capture Antibodies

1. Coat the capture antibody onto the solid phase of a microplate.

   For IgA:

   (a) Using a multichannel pipette, coat labeled 96-well microplates with 50 µl/well of a 1:500 dilution of goat anti-human IgA in Carbonate Buffer (0.1 µg/well).

   For IgM:

   (b) Using a multichannel pipette, coat labeled 96-well microplates with 50 µl/well of a 1:500 dilution of goat anti-human IgM in Carbonate Buffer (0.1 µg/well).
2. Lay the microplates flat in a moist chamber (i.e., not stacked) and incubate for 60 min at 37 °C.
3. Use the BioTek EL406 to wash the microplates (*see* **Note 4.5**).

3.3.3 Blocking Buffer

1. Using a multichannel pipette, add 200 µl of P-G-T/well.
2. Lay the microplates flat in a moist chamber (i.e., not stacked) and incubate for 60 min at 37 °C.
3. Use the BioTek EL406 to wash the microplates (*see* **Note 4.5**).

3.3.4 Addition of Serum Samples

1. Using a multichannel or single-channel pipette, add 50 µl of a 1:200 dilution of the positive-control serum and 1:200 dilution of the test serum in P-G-T in duplicate down each pair of columns. For the negative control, add 50 µl of P-G-T only (Fig. 14).
2. Incubate the microplates for 60 min, 37 °C (*plates flat in moist chamber, not stacked*).
3. Aspirate serum, fill wells with wash buffer, and allow plates to soak for 6 min.
4. Use the BioTek EL406 to wash the microplates (*see* **Note 4.5**).

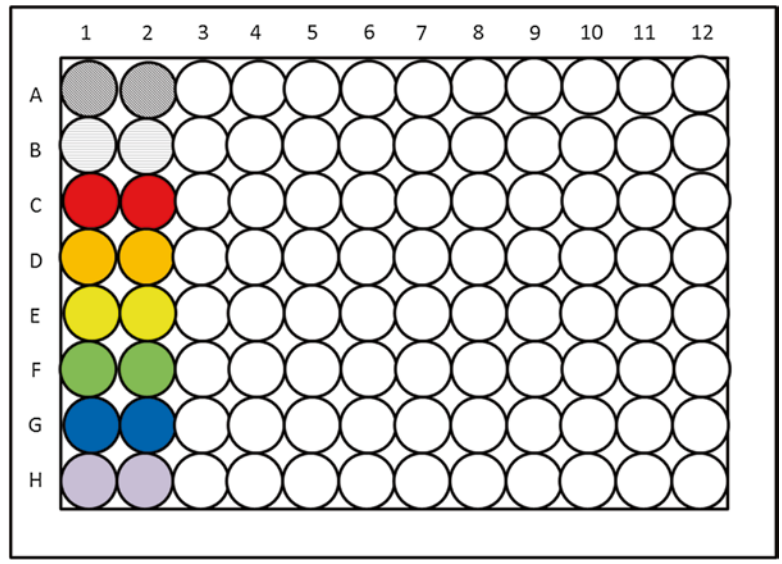

Fig. 14 Plate layout for the IgA/IgM ELISA. Wells A1-A2 and B1-B2 are designated for the positive and negative control, respectively. For simplicity, only the first 6 test specimens are indicated in C1-C2, D1-D2, E1-E2, F1-F2, G1-G2, and H1-H2. A single 96-well plate can accommodate up to 46 test specimens in duplicate

3.3.5 Addition of Type-Specific Antigen

The following steps should be performed in a biological safety cabinet if the live Sabin viruses are being used for the ELISA antigen.

1. Using a multichannel pipette and working in BSC, add 50 µl of a 1:50 dilution of each antigen in P-G-T to the appropriate plates and wells. (Antigen in columns 1–12, rows A–H).
2. Incubate the microplates overnight at room temperature (*plates flat in moist chamber, not stacked*).
3. Aspirate antigen, fill wells with wash buffer, and allow plates to soak for 6 min.
4. Use the BioTek EL406 to wash the microplates (*see* **Note 4.5**).

3.3.6 Addition of Type-Specific Monoclonal Antibodies

1. Using a multichannel pipette, add 50 µl of a 1:40,000 dilution of each monoclonal antibody in P-G-T to the appropriate plates and wells.
2. Incubate the microplates for 60 min, 37 °C (*plates flat in moist chamber, not stacked*).
3. Aspirate monoclonal antibodies, fill wells with wash buffer, and allow plates to soak for 6 min.
4. Use the BioTek EL406 to wash the microplates (*see* **Note 4.5**).

3.3.7 Addition of Horseradish Peroxidase Labeled Conjugate

1. Using a multichannel pipette, add 50 µl of a 1:4000 dilution of goat anti-mouse IgG (H+L) horseradish peroxidase labeled conjugate in P-G-T. (HRP conjugate in columns 1–12, rows A–H).

2. Incubate the microtiter plates for 60 min, 37 °C (*plates flat in moist chamber, not stacked*).
3. Aspirate conjugate, fill wells with wash buffer, and allow plates to soak for 6 min.
4. Use the BioTek EL406 to wash the microplates (*see* **Note 4.5**).

3.3.8 Addition of Substrate and Stop Solutions

1. Warm SureBlue Reserve™ Substrate to room temperature. Using a multichannel pipette, add 50 µl of substrate into each well.
2. Incubate the microplates for 15 min undisturbed at room temperature. **Do not stack plates**.
3. Warm TMB BlueStop™ Solution to room temperature. Using a multichannel pipette, add 50 µl of stop solution to each well and incubate for 10 min at room temperature.
4. Read absorbance of each plate within 10 min after addition of the BlueStop™ Solution using a standard ELISA reader with a 620 nm filter.

3.3.9 Qualitative Analysis

For qualitative analysis, negative control absorbance values from all plates corresponding to an immunoglobulin type (IgM or IgA) and antigen serotype (OPV1, OPV2, OPV3, IPV1, IPV2, IPV3) should be averaged. The procedure below uses SAS to determine the cutoff, however multiple statistical software packages are appropriate.

1. To determine the cutoff value for qualitative analysis, Perform the procedure outlined above without adding test sera (i.e., an entire plate of "blank" wells) for each polio antigen for IgM and IgA.
   (a) Ideally, "true negatives" would be used, but these are difficult to identify given the very high levels of polio immunity worldwide.
2. Use unrounded absorbance values for each isotype and antigen combination.
3. Fit the data to the Johnson family of distributions using JMP (SAS statistical package, SAS Institute).
4. Plot the cumulative density function of the Johnson distributions to show fit of data and determine 95 % confidence values (i.e., the proportion of the population with a value less than x=95 %).
5. Repeat analysis 1000 times to get an average of fits for the 95 %.
6. Apply the 95 % confidence value cutoff to each isotype and antigen serotype combination. Any absorbance value above the 95 % confidence value is considered to be "positive."

4 Notes

4.1 In-House Reference Serum for Neutralization Assays

1. The in-house reference serum should be established by measuring polio neutralizing antibody titers in a population of immunized subjects.
2. Multiple sera with high neutralization titers (≥ 7.5 $\log_2$) should be pooled to generate a high-volume control.
3. Make 100 μl aliquots of the pooled sera to be stored at −20 °C for future use. Once thawed, IHRS aliquots must be stored at 4 °C for no more than 3–4 days.
4. To prepare the IHRS for use in the serology assay, adjust the initial dilution such that the endpoint titer is reached on the 3–5 dilution on an assay plate.
5. Make the initial dilution of the IHRS and transfer to the wells that are designated for the IHRS in the randomized list (*see* Table 1).
6. When generating a new stock of IHRS, test the new stock with the old stock in parallel in the microneutralization assay (at least three times) to calibrate the new stock.

4.2 Randomization for Neutralization Assays

1. For each study, the randomization process requires a specimen number, study site, study arm, patient number, and unique ID for each specimen.
2. Each run needs to have 4–6 in-house reference sera.
3. Based on the total number of runs and the number of divisions per plate (4 divisions for 96-well plate or 14 divisions for 384-well plate), the program will also determine how many plates will be required.
4. Determine the number of unique subjects and unique study groups exist.
5. A study group is defined as a unique combination of study site and study arm for multi-site or multi-arm studies.
6. Each study group must be evenly represented in each run, so that the subjects within each study group are assigned randomly and proportionally amongst all of the runs.
7. After each subject identifier is assigned a run number, all sera associated with those subject identifiers are assigned to the same run, since comparison of sera from a single individual is usually the most important comparison (e.g., seroconversion or trends in titer).
8. Finally, plate numbers and plate positions are randomly assigned to the sera within each run.

9. Each randomized list for a study should be inspected to verify that all samples for testing are included and that the appropriate number of IHRS has been included.
10. The randomization is best performed using Visual Basic for Excel or MATLAB (available from the authors).

4.3 Poliovirus Stock Preparation and Titration for the Microneutralization Assay

4.3.1 Materials and Equipment

- T-150 tissue culture flasks (Corning, #430823).
- Single-channel pipettes:
  - Manual: LTS 20, 200, 1000 (Rainin).
  - Electronic: 10-300, 50-1000 (Biohit).
- Pipette tips.
  - Rainin: RT-L200F, RT-L1000F, RT-L10F.
  - Biohit: 350, 1000, 1200 μl.
- HEp-2(C)cells (ATCC # CCL23).
- Cell culture media.
  - Eagle's Minimum Essential Media (EMEM)(Gibco, #11095-072).
  - Penicillin/streptomycin (Gibco, #15140).
  - Fetal Bovine Serum—Optima (Atlanta Biologicals, #S12450).
  - 0.05 % Trypsin-EDTA (Gibco, #25300).
- Poliovirus stock for expansion.
- Cryovials (Wheaton, #), or equivalent.
- 96-well tissue culture, clear, sterile plates (Corning, #3997).
- Inverted microscope.
- CO_2 water-jacketed incubator (Thermo Fisher, Model 3110 or equivalent).

4.3.2 Poliovirus Stock Preparation for the Microneutralization Assay

1. Each serotype of stock poliovirus should be prepared separately.
2. For a single poliovirus, prepare confluent monolayers of Hep-2(C) cells in MEM + 10 % FBS in 150 cm^2 culture flasks at 37 °C. Prepare an additional flask to serve as an uninfected cell culture.
3. For all flasks, decant the media and wash with 10 ml of serum-free MEM.
4. Add 3 ml of MEM + 2 % FBS to each flask.
5. For the infected flask, add 3.0×10^5 $TCID_{50}$ in 100 μl of serum-free MEM.
6. Incubate the infected and uninfected flasks at 35 °C for 60 min.

7. Add 12 ml of MEM + 2 % FBS to each flask and incubate at 35 °C, 5 % CO_2 until cytopathic effect is observed (approximately 24–48 h post-infection).
8. To harvest virus, freeze the infected flask at −70 °C and thaw at room temperature. Repeat this freeze-thaw two more cycles.
9. Collect the contents of the infected flask and transfer to 15 ml conical centrifuge tube.
10. Clarify the harvested virus to remove cellular debris by centrifugation at 3000 × *g* for 5 min at 4 °C.
11. Aliquot the supernatant at 100 µl per cryovial and store at −70 °C for titration.

4.3.3 Poliovirus Stock Titration for the Microneutralization Assay

1. For each stock of poliovirus, thaw an aliquot and make tenfold serial dilutions ranging from 1:10 to $1:10^9$ in MEM + 2 % FBS.
2. Prepare a HEp-2(c) cell suspension in MEM + 10 % FBS to contain 3×10^5 cells/ml.
3. On a sterile 96-well cell culture plate, add 100 µl of each dilution to columns 1–10 for rows A/B ($1:10^6$), C/D ($1:10^7$), E/F ($1:10^8$), and G/H ($1:10^9$) (Fig. 15).
4. For the cell control, add 100 µl of MEM + 2 % FBS to columns 11 and 12 (Fig. 15).
5. Add 100 µl of HEp-2(C) cell suspension to all wells.
6. Incubate for 5 days at 35 °C, 5 % CO_2.

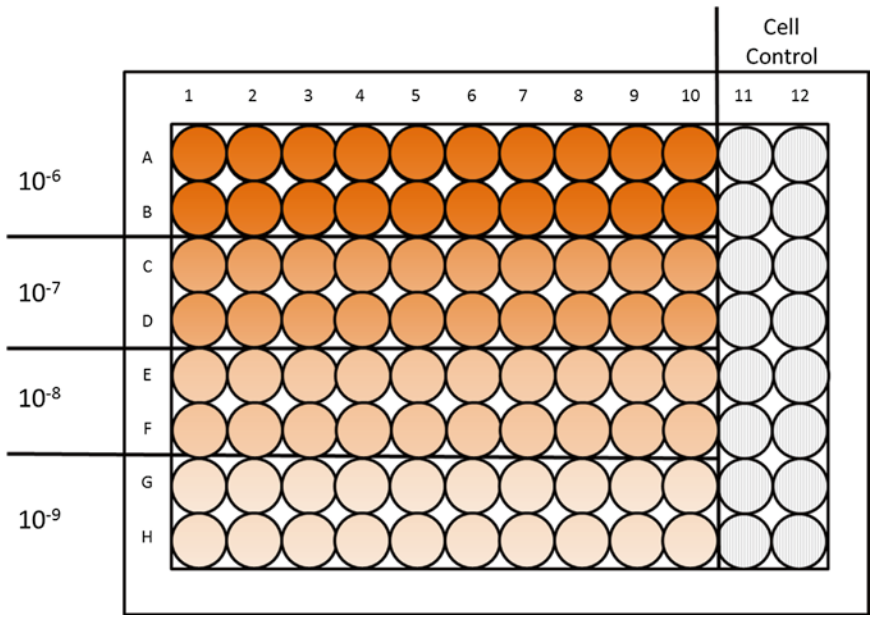

Fig. 15 Plate layout for poliovirus stock titration for the microneutralization assay

7. Count the number of wells positive for cytopathic effect for each virus dilution using an inverted microscope.
8. Calculate the virus titer using the Kärber formula:

$$Log\text{CCID}_{50} = L - d\left(S - 0.5\right), \text{where}$$

L = log of lowest dilution used in the test
d = difference between log dilution steps
S = sum of proportion of positive wells.

9. Use the titer, to calculate the dilution factor needed to obtain 100 $CCID_{50}$ in 25 µl.

4.4 Materials and Equipment for Automation of the Polio Microneutralization Assay

Most of these items may be substituted with equivalent items from other manufacturers/suppliers but alternative materials must be validated against an appropriate standard. The materials below have been validated for consistent performance in this assay.

4.4.1 Additional Consumables for Automation

- Tecan Freedom EVO conductive tips, Black, non-filtered (Phenix Research; #TRX-HTR200BK).
- 200 µl sterile tips for use with ProGroup WellPro(Phenix Research; #TRX-WP200BRS).

4.4.2 Equipment and Automation

- CO_2 water-jacketed incubator (Thermo Fisher Model 3110 or equivalent).
- WellPro 4000 CE (ProGroup).
- EL406 plate washer and dispenser (BioTek).
- MicroFlo dispenser (BioTek).
- Tecan Evo100 or equivalent (Tecan).
- TC20 Automatic Cell Counter (Bio-Rad).
- Victor X4 Multimode plate reader (PerkinElmer) or equivalent.

4.4.3 Protocol

Optional: 1 Day Before Test Run

1. Use the MicroFlo reagent dispenser to add 25 µl MEM 2 % FBS to the test plates and the back titration plates for PV1, PV2 and PV3.
   (a) For the cell control plate, a total of 50 µl MEM + 2 % FBS should be added to each well.
2. Use the Tecan Evo100 to make the triplicate sample plates (Fig. 4a, b) by transferring 100 µl of the test serum in a deep-well plate (from **step 1**) in triplicate to a triplicate sample plate.
   (a) Using electronic multichannel pipette, transfer 25 µL of test serum from triplicate sample plate (generated in **step 3**) to row A of three test plates (one for each serotype) (Fig. 4c).

Day of Run

1. Use the WellPro to perform twofold serial dilutions (Fig. 5).
   (a) The WellPro will transfer 25 µl of the diluted serum in row A to row B, then from row B to row C, etc.
   (b) For row H, 25 µl will be aspirated and discarded with the tips, to maintain the correct total volume.
   (c) For each transfer, the program will mix the diluted serum a total of four times.
   (d) Using this program will produce serum dilutions ranging from 1:8 to 1:1024.
   (e) This step generates the test plates, ready for addition of virus and cells.
2. Use the MicroFlo to add 25 µl of 100 $CCID_{50}$ of relevant poliovirus to all wells in the diluted serum test plates. Use a different sterile dispensing cartridge for each virus.
3. Use the MicroFlo to add 25 µl of prepared cell suspension to each well of every plate.

Plate Washing and Staining

Due to the chances of aerosolization of virus in media from the microneutralization plates, this step should be done in a biological safety cabinet.

(a) After 5 days incubation, use the washer to aspirate and add 50 µl of a 0.05 % crystal violet solution to all wells (Fig. 8).
(b) Incubate for a minimum of 40 min at room temperature.
(c) Using the washer, aspirate the crystal violet stain then fill all wells with 250 µl of tap water and aspirate.
(d) This process will be repeated three more times to completely remove any excess crystal violet stain in the test plates prior to reading.

4.4.4 Data Collection

1. Read the crystal violet-stained microplates with an ELISA reader at a 595 nm wavelength.
2. To determine the absorbance cutoff, determine 80 % of the average absorbance value for the cell control plate.
3. Absorbance data from plate reader can be processed using macros written in Visual Basic for Microsoft Excel.

4.5 Polio IgA/IgM Capture ELISA Wash Procedure

4.5.1 Procedure

1. Fill wash reservoir with the prepared wash buffer.
2. The program will aspirate the 96-well plate and dispense 250 µl of wash buffer to each well.
3. Soak the microplates for 6 min.
4. Repeat this program three more times to complete the washing procedure.
5. Tap plates on a paper towel to remove residual liquid.

4.6 Cell Culture Media Preparation

4.6.1 Materials and Equipment

- Cell culture media
  - Eagle's Minimum Essential Media (EMEM)(Gibco, #11095-072).
  - Penicillin/streptomycin (Gibco, #15140).
  - Fetal Bovine Serum—Optima (Atlanta Biologicals, #S12450).
  - 0.05 % Trypsin-EDTA (Gibco, #25300).
- Nalgene 500 ml, 0.20 μm filter (Nalgene, #450-0020).

4.6.2 Procedure

1. FBS must be inactivated at 56 °C for 30 min and filtered with a Nalgene 0.2 μm filter prior to media preparation.
2. Prepare MEM+ 2 % FBS and MEM + 10 % FBS:
   (a) Add 1 ml of streptomycin/penicillin to 1000 ml of EMEM (0.1 % final concentration).
   (b) Add 20 ml (2 %) or 100 ml (10 %) of fetal bovine serum to 1000 ml bottle of EMEM.
   (c) For serum free MEM, add no fetal bovine serum.
   (d) MEM + 10 % FBS is used for maintaining HEp-2(C) cells and generating the cell suspensions.
   (e) MEM + 2 % FBS is used for filling the assay plates, diluting sera, and diluting virus.