Chapter 4

Isolation and Characterization of Poliovirus in Cell Culture Systems

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Abstract

The isolation and characterization of enteroviruses by cell culture was accepted as the "gold standard" by clinical virology laboratories. Methods for the direct detection of all enteroviruses by reverse transcription polymerase chain reaction, targeting a conserved region of the genome, have largely supplanted cell culture as the principal diagnostic procedure. However, the World Health Organization's Global Polio Eradication Initiative continues to rely upon cell culture to isolate poliovirus due to the lack of a reliable sensitive genetic test for direct typing of enteroviruses from clinical specimens. Poliovirus is able to infect a wide range of mammalian cell lines, with CD155 identified as the primary human receptor for all three seroytpes, and virus replication leads to an observable cytopathic effect. Inoculation of cell lines with extracts of clinical specimens and subsequent passaging of the cells leads to an increased virus titre. Cultured isolates of poliovirus are suitable for testing by a variety of methods and remain viable for years when stored at low temperature.

This chapter describes general procedures for establishing a cell bank and routine passaging of cell lines. While the sections on specimen preparation and virus isolation focus on poliovirus, the protocols are suitable for other enteroviruses.

Key words Poliovirus, Enterovirus, Virus isolation, Cytopathic effect, Cell culture, Continuous cell lines, Cryopreservation of mammalian cells, Cell bank, Extraction of clinical specimens, Immunofluorescence

1 Introduction

The devastating impact of worldwide poliomyelitis outbreaks during the twentieth century led to intensive study of the causative agent, poliovirus. The description by Enders, Weller and Robbins in 1949 of the propagation of poliovirus type 2 Lansing strain in non-neural tissue was a breakthrough, not only for research into poliomyelitis but also for diagnostic virology [1]. The propagation of poliovirus was confirmed by the induction of paralysis in mice and monkeys after intracerebral inoculation of passaged cultures and neutralization of poliovirus activity by antiserum specific for the Lansing strain. The following year the trio published their findings that cytopathogenic effect, later termed cytopathic effect (CPE), of poliovirus

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propagation could be observed microscopically as degeneration of tissue cultures [2]. In a subsequent series of papers, the three authors refined the procedures to cultivate poliovirus in tissue culture from stool specimens using antibiotics and roller cultures that obviated confirmation of virus propagation by more complex techniques involving animals [3, 4]. In 1954, the three authors received The Nobel Prize in Physiology or Medicine for this seminal work.

Further milestones in the history of cell culture procedures occurred in the 1950s, including the introduction of trypsin to generate single cell suspensions for subculture, the establishment of the first human cell line, HeLa, precluding the reliance on primary cell culture, and the development of defined culture media [5]. All of these events were necessary for the large scale propagation of poliovirus in vitro and enabled the development of various polio vaccines, with the inactivated and live-attenuated products developed by Salk and Sabin, respectively, the most well-known.

The World Health Assembly established the Global Polio Eradication Initiative in 1988. The World Health Organization (WHO) polio eradication program is built upon maintaining high vaccine coverage with the Sabin oral polio vaccine to stop person-to-person transmission of poliovirus, surveillance for cases of acute flaccid paralysis (polio-like illness) in children and the testing of stool specimens from children presenting with acute flaccid paralysis by virus culture in a laboratory accredited by WHO. Virus culture of stool specimens presents challenges due to toxicity and an abundance of other microorganisms. Furthermore, non-polio enteroviruses can be isolated by culture from up to 20 % of stool specimens of acute flaccid paralysis cases, depending on the geographical location, and their cytopathic effect in virus culture cannot be distinguished from that caused by poliovirus [6, 7]. All cell culture isolations of enterovirus, including poliovirus, require confirmation by techniques such as immunofluorescence, antisera neutralization, ELISA, and reverse transcription polymerase chain reaction (RT-PCR).

Continuous mammalian cell lines should be ordered from a reliable source that can provide evidence of cell authentication [8]. With careful handling and use of appropriate equipment, cell lines can be stored for many years in liquid nitrogen and revived for routine passaging as required. The procedures in this chapter describe the receipt of cell lines, cell banking, specimen extraction for virus culture and virus isolation for poliovirus and other enteroviruses.

2 Materials

1. Cell lines. While poliovirus types 1, 2 and 3 can be cultured in a wide variety of cell lines, the WHO recommends use of the L20B and RD mammalian cell lines:
   (a) L20B. Mouse fibroblast L-cells genetically engineered for cell surface expression of the human poliovirus receptor,

CD155 [9]. The cell line facilitates the isolation of poliovirus in preference to non-polio enteroviruses but some strains of Coxsackie virus A (CV-A), in particular CV-A8 and CV-A10, are capable of growth in L20B with a characteristic enterovirus cytopathic effect (CPE) [10, 11]. Adenoviruses and reoviruses are also capable of infecting L20B with a CPE that is distinct from enteroviruses.

(b) RD. The human rhabdomyosarcoma (RD) cell line has been utilized for the growth of a wide range of enteroviruses. RD cells are particularly useful for the isolation of type A Coxsackie viruses and echoviruses but strains of type B Coxsackie virus grow poorly. Polioviruses can grow to a titre of greater than 10^8 $CCID_{50}$/50 μl in the RD cell line. The suffix "-A" in RD-A denotes the cell line was sourced from a member of the WHO Global Polio Reference Laboratory network.

2. Cell culture media (*see* **Note 1**). Poliovirus growth in continuous cell lines may be in an open system with an atmosphere that includes CO_2 or in a closed system with the lid of the flasks and tubes tightened. Complete medium consists of Eagle's minimum essential medium (MEM) as the base constituent and is supplemented with serum, balanced salts (Earle's or Hank's), buffering agents (sodium bicarbonate and HEPES), L-glutamine (an essential amino acid), and antibiotics (Tables 1 and 2). Growth medium includes 10 % fetal bovine serum as a source of nutrients for metabolism and cell division. The proportion of fetal bovine serum is reduced to 2 % in holding or maintenance media for sustenance while limiting cellular division. The specific media requirements of cell lines other than L20B and RD should be confirmed to optimize growth of both the cell line and virus.

Table 1
Eagle's complete MEM for poliovirus culture in the presence of CO_2 (open system)

Component	Growth medium	Maintenance medium
Eagle's minimum essential medium (Earle's salts with phenol red, no bicarbonate)	83.5 ml	90.5
L-glutamine (200 mM)	1.0 ml	1.0 ml
Fetal bovine serum (heat inactivated)	10.0 ml	2.0 ml
Sodium bicarbonate, 7.5 % solution	3.5 ml	4.5 ml
HEPES 1 M	1.0 ml	1.0 ml
Penicillin (1×10^4 U/ml)/streptomycin (10 mg/ml) solution	1.0 ml	1.0 ml
Total volume	100 ml	100 ml

Table 2
Eagle's complete MEM for poliovirus culture in the absence of CO_2 (closed system)

Component	Growth medium	Maintenance medium
Eagle's minimum essential medium (Earle's salts, no bicarbonate)	85.5 ml	92.5
L-glutamine (200 mM)	1.0 ml	1.0 ml
Fetal bovine serum (heat inactivated)	10.0 ml	2.0 ml
Sodium bicarbonate, 7.5 % solution	1.5 ml	2.5 ml
HEPES 1 M	1.0 ml	1.0 ml
Penicillin (1×10^4 U/ml)/streptomycin (10 mg/ml) solution	1.0 ml	1.0 ml
Total volume	100 ml	100 ml

3. Sterile cell culture flasks: 25 cm^2, 75 cm^2, 175 cm^2.
4. Sterile tubes with externally threaded screw capped lids:
   (a) Polypropylene 1.5 ml, 50 ml.
   (b) Polycarbonate 5 ml.
   (c) Cryogenic 1.8 ml ampoules.
   (d) Sterile cell culture tubes.
5. Sterile nontoxic polyethylene transfer pipettes.
6. Sterile serological pipettes: 5, 10, and 25 ml.
7. Hemocytometer (e.g., improved Neubauer) and glass coverslips.
8. Gloves. Examination gloves are made from latex or nitrile that can be of varying quality. Select a brand that will not easily tear and that does not cause skin irritation from repeated use.
9. Cell freezing container. Insulated containers designed to facilitate the freezing of cell lines at a controlled rate are available.
10. Sterile wooden tongue depressor.
11. Laboratory sealing film.
12. Glass beads of 2 and 5 mm diameter, used to process postmortem and stool specimens, respectively. The beads should be washed and dried before use.
13. Microscope slide staining jar.
14. Anti-poliovirus blend mouse antibodies and anti-mouse IgG-FITC labeled secondary antibody.

15. Equipment:
    (a) Variable volume pipettes and filter tips: 2–20 and 20–200 µl.
    (b) Inverted microscope.
    (c) Refrigerated bench centrifuge with buckets suitable for cell culture tubes and 50 ml tubes.
    (d) Biological safety cabinet class II.
    (e) Liquid nitrogen storage container and personal protective equipment for cryogenic procedures.
    (f) Refrigerator, –20 and –80 °C freezers.
    (g) Bench top vortex.
    (h) Mechanical shaker.
    (i) Warm air incubator or CO_2 incubator with shelving and a mechanical roller.
    (j) Plastic racks to hold tube cultures.
    (k) Hand-held pipette aid for serological pipettes.
16. Solutions:
    (a) Eagle's minimum essential medium (Earle's or Hank's salts with phenol red, no bicarbonate).
    (b) L-glutamine (200 mM). An essential amino acid that is unstable at 36 °C.
    (c) Fetal bovine serum (FBS) (*see* **Note 2**).
    (d) Sodium bicarbonate (7.5 %).
    (e) HEPES (1 M). Provides additional buffering capacity to sodium bicarbonate at physiological pH.
    (f) Penicillin (10^4 U/ml) and streptomycin (10 mg/ml).
    (g) Sterile phosphate buffered saline (PBS) solution. Available commercially with (complete PBS) and without (incomplete PBS) calcium and magnesium ions. Complete PBS is used for specimen extraction with the divalent cations stabilizing the enterovirus particles. Incomplete PBS is used when passaging cell lines to avoid the divalent cations interfering with the activity of the EDTA–trypsin solution.
    (h) Trypsin–EDTA solution.
    (i) Ethanol.
    (j) Chloroform. Stabilized with ethanol and use within 2 years of opening.
    (k) Trypan blue stain (0.4 %).
    (l) Dimethyl sulfoxide (DMSO). Use within 3 months of opening. Multiple ampoules of smaller volume can be purchased rather than a larger bottle.

(m) Acetone.

(n) Glycerol.

(o) Disinfectant: a number of commercial disinfectants are available that have proven virucidal activity. Alcohols do not inactivate poliovirus.

3 Methods

3.1 Establishing a Cell Bank for the Isolation of Poliovirus Using Continuous Mammalian Cell Lines

The proper handling and storage of continuous cell lines is important to maintain their sensitivity to poliovirus infection (*see* **Note 3**). Cell lines should be stored either submerged or within the vapor phase of liquid nitrogen to retain long-term viability. Storage in a −80 °C freezer is not recommended. All handling and manipulations with the cell lines described in this chapter should be performed within a class 2 biological safety cabinet (BSC). While a cell culture laboratory is considered a "clean" environment when compared to a virus isolation laboratory that processes clinical specimens, many cell culture reagents such as serum, trypsin and the cell lines are derived from animal sources and thus, all items should be considered as potentially infectious. Appropriate personnel protective equipment should be worn when handling cell culture material.

Cell lines should be received from a reliable commercial or laboratory source. The supplier should provide the history of the cell line indicating its original source, test results for the presence of adventitious agents such as mycoplasma, the culture media and supplements recommended for growth and maintenance of the cell line and the seeding level for routine subculture or passage. The supplier should also specify the absolute passage number and confluency of the cells at the time of shipment, the culture media in the flask and whether the cells were grown in an open or closed system.

Continual passage of cell lines will result in genotypic and phenotypic variation from the cell line originally received. The intention when establishing a new cell bank is to expand the number of cells exponentially for storage of multiple ampoules in liquid nitrogen but in as few passages as possible so as to retain the characteristics of the cell line. Once a cell line has been revived from liquid nitrogen it should only be passaged a set number of times to retain the cell phenotype and also minimize the risk of contamination with mycoplasma or other agents through repeated handling. The number of ampoules required for storage in liquid nitrogen will depend upon how routinely the cell line will be used. WHO recommend that L20B and RD cell lines should not be passaged more than 15 times or beyond 3 months after revival from liquid nitrogen, whichever occurs first [12]. For example, if a cell line is passaged once a week, a fresh ampoule should be revived every 3 months or 12 passages. This means four ampoules will be required every year and preparation of a cell bank of 50 ampoules will last

for more than 7 years. If a cell bank needs to be replenished it will require two to three passages to expand the number of cells and the master cell bank passage number will be further from the original as a consequence.

1. Upon receipt of the cell line from the sending laboratory, inspect the flask for cracks or evidence of leakage and examine the condition of the cells microscopically (*see* **Note 4**). Record the morphology and confluency, which should also be reported to the sender.
2. Incubate the flask overnight at 36 °C to acclimatize the cells.
3. Replace the culture media the next day according to the confluency: <75 % use growth media, >75 % use maintenance media. Return the flask to the incubator until the confluency is >80 %, but do not allow to reach 100 % so as to maintain exponential cell growth, at which point the cells are passaged.
4. Passage the cells by preparing a single cell suspension after incubation with trypsin–EDTA, which detaches cells from the surface of the flask. Tip off the culture media into a waste discard container and wash the monolayer twice with incomplete PBS that does not contain the divalent cations, magnesium and calcium (*see* **Note 5**).
5. Add sufficient trypsin–EDTA to just cover the cell monolayer; approximately 0.5 ml for a 25 cm^2 flask and 1 ml for a 75 cm^2 flask. Gently roll the flask from side to side to ensure the trypsin–EDTA covers the cells and place in a 36 °C incubator to assist the enzymatic activity of trypsin. Remove the flask from the incubator every minute to observe the cell monolayer under a microscope. Each time return the flask to the incubator until the cells round up and start to detach from the surface (*see* **Note 6**).
6. Add 10 ml of growth media and detach any remaining cells by repeatedly flushing the surface gently using a transfer pipette. Transfer the cell suspension to a 50 ml tube and disperse cell aggregates by gently pipetting up and down with a transfer pipette.
7. Perform a cell count using a hemocytometer (*see* **Note 7**).
   (a) Clean the surface of the counting chamber and the glass coverslip with ethanol to remove any grease or dust and polish dry. Place the coverslip over the two sets of gridlines on the chamber. Dilute 0.2 ml of the single cell suspension with 0.2 ml of Trypan blue and mix well (*see* **Note 8**).
   (b) Using a micropipette with a narrow bore tip, gently expel 10 µl of the dye–cell suspension to one side of the counting chamber by touching the tip to the underside of the coverslip. Remix the dye–cell suspension to ensure independent sampling and load 10 µl to the other side of the counting chamber.

(c) Record the number of viable (unstained) cells in one half of the hemocytometer and repeat the counting procedure for the second half (*see* **Note 9**). Determine the mean of the two cell counts. Check that the individual counts are within 20 % of the mean, to indicate accurate sampling and prepare a fresh Trypan blue cell suspension if they are not.

(d) The viable cell concentration per ml is determined by the formula:

$$iCC = t \times df \times 1/4 \times 10^4$$

iCC = initial cell concentration per ml

t = total cell count of both sides of the counting chamber

df = dilution factor of cell suspension with Trypan blue

1/4 = correction factor for mean number of cells per corner grid

10^4 = conversion factor of counting chamber

Example: $t = 480$; $df = 2$; $iCC = 480 \times 2 \times 1/4 \times 10^4 = 2.4 \times 10^6$ per ml.

8. Determine the dilution (d) required to seed the new flasks by dividing the working cell concentration (wCC) by the initial cell concentration (iCC) from **step 6d**.

   $wCC = 2 \times 10^5$ cells per ml

   Example: $d = 2 \times 10^5 / 2.4 \times 10^6$ per ml $= 1/12$

   The working cell concentration is prepared by adding one ml of the cell suspension to a new flask and diluting with 11 ml of growth media.

9. Seed as many flasks as required with the working cell concentration, add the appropriate volume of growth media and incubate at 36 °C. It is preferable to expand the number of cells by using large cell culture flasks (175 cm^2) to minimize the number of passages required to establish the cell bank.

10. Assess the confluency of the cells by microscopic observation each day. When the confluency is 80–90 %, detach cells from the flasks with trypsin–EDTA. Perform a cell count and determine the total number of cells harvested from the flasks by multiplying the cell concentration by the volume of the cell suspension. Determine the number of ampoules that can be cryopreserved at a cell concentration of 5×10^6 cells per ml (*see* **Note 10**). If the number of ampoules is less than required seed a further set of cell culture flasks and repeat **step 8**.

11. Cryopreservation of cell lines. The general rule for the cryopreservation of continuous cell lines is to freeze slowly, thaw quickly and dilute slowly. A cryoprotectant such as dimethyl sulfoxide (DMSO) is required to prevent ice crystal formation that would rupture cells during freeze-thawing. DMSO is water mis-

cible and a powerful solvent that readily penetrates cell membranes and also human skin. Chemical-resistant gloves or two layers of examination gloves should be worn when handling DMSO and immediately changed and hands washed if the chemical is spilt on the gloves. DMSO is hygroscopic (absorbs moisture from the air) and should be used within 3 months of opening. It is recommended to purchase individual ampoules of a small volume (10 ml) rather than a single large bottle. The serum concentration in the growth medium used for cryopreservation of cells can be increased to 20 % to assist with cell recovery.

(a) Label the number of ampoules required for cryopreservation, determined at **step 9**, with the cell line, passage number, and date of cell banking. Ensure the ampoules are permanently labeled either with an ethanol-resistant marking pen by hand or with cryogenic labels made for the purpose. Uncap the ampoules and stand upright in a rack.

(b) Prepare sufficient freezing mix for the total volume of cells available consisting of growth media with 20 % serum and supplemented with 10 % DMSO. Chill the freezing mix prior to use.

(c) Centrifuge the cell suspension at $1000 \times g$ for 5 min. Immediately discard the supernatant and drain the residual media on tissue. Cap and hold the top of the tube with one hand while flicking the bottom of the tube hard with the other hand to dislodge the cell pellet.

(d) Add the freezing mix and gently swirl the cells checking that they are uniformly suspended and, if not, gently mix with a transfer pipette.

(e) Add 1 ml of cell suspension in freezing mix per labeled ampoule and securely cap the tube. The ideal cooling rate for the cells is 1 °C/min, which can be achieved in a number of ways. Specially designed apparatus that fit within the neck of a liquid nitrogen container can be used to lower the cells through the gaseous phase at a controlled rate. Insulated containers with either sealed coolant or requiring the addition of alcohol are available. Alternatively, the cell cooling rate can be slowed by insulating the cells within polystyrene foam and a towel. Do this by placing the tubes in a polystyrene foam rack and cover with a second rack. Tape the racks together and wrap in a large, thick bath towel and secure by running tape around the bundle ensuring the ampoules are kept upright during the process. Using either commercial containers or racks wrapped in towels to control the cooling rate, transfer the cells to a −20 °C freezer for 2 h, followed by overnight equilibration to −80 °C. The next day remove the ampoules

from the insulated material and transfer to liquid nitrogen (*see* **Note 11**).

(f) Within a week, retrieve a single ampoule to assess the viability of the cells stored in liquid nitrogen. If the cell recovery is low a new cell bank should be prepared.

(g) Maintain an accurate and up to date inventory of the cell type, number of ampoules and location stored in liquid nitrogen.

12. Retrieval of cell lines from liquid nitrogen. Cells must be thawed quickly to prevent intracellular ice crystal formation leading to cell death. Have everything in readiness in the BSC before retrieving the cells from liquid nitrogen.

(a) Label the cell culture flasks. Have a plastic beaker with warm water and a spray bottle of 70 % ethanol on hand.

(b) Retrieve the ampoules from liquid nitrogen checking the label to confirm the details and later update the inventory.

(c) Transfer to the cell culture laboratory in a plastic container rather than a glass beaker in case liquid nitrogen entered the ampoule and ruptures on warming.

(d) In the BSC, hold the ampoule and rapidly move the frozen cells backwards and forwards in the beaker of warm water.

(e) Place the ampoule with the thawed cells on tissue and spray the outside of the ampoule with 70 % ethanol.

(f) Open the ampoule and transfer the thawed cells to a labeled flask.

(g) Add growth media to the cells dropwise while gently moving the flask backwards and forwards to facilitate dilution of the cells. The gradual dilution of DMSO avoids osmotic shock that will reduce cell recovery. The rate of addition can be slowly increased once more than 2–3 ml of media has been added to the flask. The cells should be diluted in a final volume 10–20 times that stored in liquid nitrogen to reduce the cytotoxic effects of DMSO.

(h) Cap the flask and incubate at 36 °C.

(i) The next day, observe the cell morphology under a microscope and record the confluency of the monolayer. Discard the media containing the diluted DMSO and replace with fresh growth media in readiness for subculturing and routine use.

13. Mycoplasma testing. Mycoplasma are ubiquitous intracellular microorganisms of human and animal origin that cannot be observed by microscopy but can modify a cell's phenotype. Continuous cell lines can become infected with mycoplasma

from the operator, other cell lines and reagents from animal sources such as serum and trypsin. Regular screening of routine cell cultures, at least once per revival from liquid nitrogen, should be performed by microbiological culture, fluorescent staining, PCR, or ELISA using either published protocols or commercial kits. Cell lines positive for mycoplasma should be discarded.

3.2 Routine Passaging of Continuous Mammalian Cell Lines and Preparation of tube Cultures for Virus Isolation

After retrieval from liquid nitrogen stocks, cell lines are maintained in flasks and used to prepare tube cultures for virus isolation (*see* **Note 12**). To reduce genotypic and phenotypic variation of the cell line from that originally supplied and the likelihood of contamination by mycoplasma or other agents, do not passage more than 15 times or beyond 3 months after retrieval from liquid nitrogen, whichever occurs first. Depending on the number of specimens received for processing, the tube cultures are prepared weekly or twice weekly.

1. Microscopically assess the confluency of the cell monolayer in flasks. The confluency of the monolayer should be less than 90 % so that the cells are within the exponential growth phase and should not overgrow before the next passage.
2. Remove the cells from the flask with trypsin–EDTA and perform a cell count following **steps 4**–**7** in Subheading 3.1.
3. Seed the required number of flasks with cell suspension in growth media according to Table 3. Record the passage number on the flask (*see* **Note 13**). Incubate the flasks at 36 °C until the cell monolayer is approximately 80 % confluent after 5–7 days when the growth media should be replaced with maintenance media unless the cells will be split on that day.
4. Seed the required number of culture tubes for poliovirus isolation according to Table 3. The seeding level should be adjusted based on experience to achieve a cell confluency of approximately 75 % after 48 h in growth media.

Table 3
Volumes and seeding levels for culture of the L20B and RD cell lines

Cell culture container	Volume (ml)	Seeding level (total cell number)
Tube: 125 × 16 mm	1	1×10^5
Small flask: 25 cm^2	10	1×10^6
Medium flask: 75 cm^2	30	2.5×10^6
Large flask: 175 cm^2	75	5×10^6

Determine the dilution (d) required to seed the tubes by dividing the working cell concentration (wCC) by the initial cell concentration (iCC) determined by the cell count.

$wCC = 1 \times 10^5$ cells per ml

Example: $d = 1 \times 10^5 / 2.4 \times 10^6$ per ml $= 1/24$

The working cell concentration is prepared by adding one ml of the cell suspension to a new flask and diluting with 23 ml of growth media.

5. Uncap the cell culture tubes and aliquot 1 ml of the cell suspension into each tube using a sterile graduated transfer pipette.
6. Recap the culture tubes and place in a rack with the orientation marker turned to the top (*see* **Note 14**).
7. Place the tube rack in a 36 °C incubator.
8. Once the cells reach the required confluency change the culture media in the tubes with maintenance media and again before inoculation with specimen extract, unless this is on the same day. The tubes may be used for virus isolation for 5–7 days after the original seeding.

3.3 Preparation of Clinical Specimens for the Isolation of Poliovirus

The main mode of poliovirus transmission is via the fecal–oral route and the preferred type of specimen for virus isolation is stool. While poliovirus can be isolated from throat swabs and nasopharyngeal aspirates for up to two weeks early on in the infection, the virus is shed for up to 6 weeks in stools. To optimize the isolation of poliovirus, it is recommended to collect two stool specimens more than 24 h apart—due to intermittent virus shedding—and within 14 days of the onset of symptoms when the virus titre is greatest. While it is possible to isolate poliovirus from rectal swabs, whole stool specimens are preferred as the quantity of virus, and hence test sensitivity, is likely to be greater within a whole specimen.

A period of viraemia can lead to replication of poliovirus in the central nervous system and present as paralytic poliomyelitis, the clinical condition the general public most associate with the virus. Despite an association with neurological disease, poliovirus isolation from CSF is uncommon and considered not worth attempting if stool specimens are available. Poliovirus infection can be fatal, especially with bulbar paralysis involving the respiratory muscles, and can also present as encephalitis and meningitis. This section describes the preparation of stool specimens, swabs, and postmortem tissue for poliovirus culture.

1. Turn on the class II BSC and allow the airflow to equilibrate. Maintain the minimal number of items required for the procedure in the cabinet. Never place items across the front grill and do not make rapid movements within the cabinet or when

moving items into and out of the cabinet as this disrupts the airflow curtain that provides protection to the worker. Always manipulate fluids slowly and gently to avoid creating aerosols.

2. Prepare a container for disposal of potentially infectious waste (*see* **Note 15**).
3. Organize the work area so that sterile reagents and samples are to one side of the cabinet, waste container to the other and specimens to be processed located centrally.
4. Cabinets should be periodically tested for filter integrity (e.g., oil mist test) and operator protection (e.g., potassium iodide release test). Testing should conform to international standards such as AS 2252 (Australia), EN 12469 (European), JIS K 3800 (Japan), and NSF 49 (USA) and should be repeated when the cabinet is moved or the laboratory layout altered, particularly involving changes to the air flow within the room.
5. Clean and decontaminate the cabinet inner surfaces (both horizontal and vertical) after every working session and periodically (e.g., once per month) decontaminate and clean the tray under the BSC working surface.
6. Preparation of stool specimens. Ideally 5 g of stool specimen should be collected and 2 g (approximately the size of a small thumbnail joint) processed for virus culture (*see* **Note 16**).
   (a) Label the original specimen containers and individual 50 ml tubes with unique identifying codes. The use of 50 ml tubes is recommended to ensure adequate mixing of the extract. Also label two 5 ml tubes for long-term storage of the stool extract at **step** (**j**).
   (b) Add 10 ml complete PBS, approximately 1 g of 5 mm glass beads and 1.0 ml chloroform per tube and reseal the caps (*see* **Note 17**). If 1 g or less of stool specimen is received, the quantities may be halved to avoid dilution of any viruses present; use 5 ml complete PBS, 0.5 g 5 mm glass beads, and 0.5 ml chloroform.
   (c) Working with one specimen at a time, transfer approximately 2 g of stool specimen to a labeled tube with a sterile wooden tongue depressor. The tongue depressor may be broken in half lengthways and used for two specimens. Ensure that the unique identifying code of the original sample matches the code on the 50 ml tube.
   (d) Retain the remaining original specimen for storage at –20 or –80 °C.
   (e) Close the tube securely and wrap laboratory sealing film in a clockwise direction around the lid to prevent leakage and loosening of the cap.

(f) Vortex the specimen extract for 1 min to assist with dispersal of the specimen.

(g) Shake tubes vigorously using a mechanical shaker for 20 min (*see* **Note 18**).

(h) Ensure the caps of the 50 ml tubes are secure before transferring to centrifuge buckets that can be sealed with an aerosol-resistant lid. Spin for 20 min at 1500 × *g* in a refrigerated centrifuge at 4 °C.

(i) Transfer the centrifuge buckets to the BSC and open the aerosol-resistant lids. Check for leakage and decontaminate all surfaces if necessary.

(j) Working with one tube at a time, transfer the upper aqueous phase into two pre-labeled 5 ml storage vials from (**a**). Avoid disturbing the interface and the lower phase containing the chloroform. If the upper phase appears turbid or a well-defined interface was not observed after centrifugation, transfer the upper phase to a clean 50 ml tube, relabel and repeat the extraction by adding 1 ml chloroform and follow **steps** (**e**)–(**j**).

(k) Store one tube of stool extract at −20 °C as a backup and the other at 4 °C ready to inoculate onto cell monolayers.

(l) Discard the biological waste material, remove items from the BSC and decontaminate the work surface with disinfectant.

7. Preparation of swabs and postmortem specimens. Poliovirus can be isolated from throat, nose and rectal swabs, although they are considered not to be ideal specimens for virus isolation due to lower virus yield compared to whole stool and the shorter duration of virus replication in nasal and pharyngeal tissue. Postmortem specimens such as brain, bowel, and spinal cord may be referred from fatal cases of suspected poliovirus infection. A simple extraction method for clinical specimens is to pulverize the tissue or swab with 2 mm glass beads rather than using mortar and pestle.

(a) Working in a BSC, place postmortem tissue in a sterile petri dish and slice into small pieces with a sterile scalpel. Transfer the tissue slices to a small sterile bottle containing glass beads of 2 mm diameter and 2 ml complete PBS and secure the lid. For swabs, place the item in a bottle containing 2 mm glass beads and snap the shaft so that the swab fits within the bottle. Add 2 ml complete PBS and secure the lid.

(b) Vortex the bottle vigorously for 3 min.

(c) Transfer the liquid to a labeled 1.5 ml tube and centrifuge for 1 min at 15,000 × *g*.

(d) Transfer the supernatant to a second labeled 1.5 ml tube and store at 4 °C until ready to inoculate the cell monolayers and at −20 or −80 °C for long-term storage.

3.4 Virus Isolation

The three serotypes of poliovirus grow readily in a wide variety of continuous mammalian cell lines but the WHO recommends using L20B and RD, which are sensitive to poliovirus infection and enables the standardization of techniques and comparison of results between laboratories.

Poliovirus replication produces an observable cytopathic effect in sensitive cell lines that is characteristic of enteroviruses.

1. Microscopically examine recently prepared tube cultures to ensure the cell monolayer has the appearance of normal morphology and is approximately 75–90 % confluent. Refer to Subheading 3.2.
2. Discard the media and replace with 1 ml maintenance media.
3. Label two tubes each of RD and L20B cells with a unique code for each specimen extract to be inoculated and indicate the history of the virus culture (*see* **Note 19**). The use of two tubes per cell line will increase the sensitivity of poliovirus isolation. Label an uninoculated negative control tube of each cell line with the passage number and the date of virus culture (*see* **Note 13**). Ensure labeling will not obscure the monolayer when observed under a microscope.
4. Place the labeled cell culture tubes upright in a rack and in the same order as the specimen extract tubes in another rack.
5. Working with one specimen at a time, remove the cap of the specimen extract tube and the caps of the appropriately labeled culture tubes. Using a micropipette and plugged filter tips, inoculate each culture tube with 0.2 ml of specimen extract (*see* **Note 20**). Re-cap all tubes and repeat for each specimen.
6. Place the inoculated L20B cells and negative cell control tube in a stationery rack, the same as used for the seeding of the cell line. Place the RD cells and negative cell control tube in a tube-holder roller drum assembly that turns at approximately 0.2 rpm. Both the virus culture rack and drum should be inclined approximately 5° towards the back compared to a level horizontal surface so the media covers the cell monolayer at the bottom of the tube and does not flow into the cap.
7. Examine the tube cultures each day under a microscope. Start with the negative control tube to check that the appearance of the cell morphology is normal. Carefully examine the entire cell monolayer of the tubes inoculated with the specimen extract for the appearance of CPE (Figs. 1 and 2). Record the appearance of the cell monolayer as negative or grade the CPE according to the proportion of the cell monolayer that is

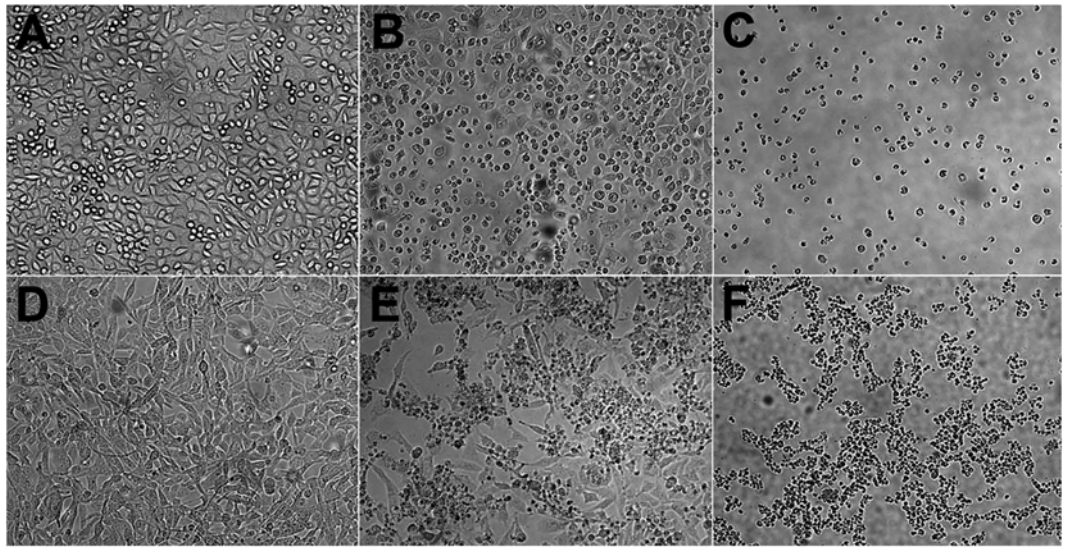

Fig. 1 L20B and RD cell lines. The L20B (panels **a**, **b**, and **c**) and RD (panels **d**, **e**, and **f**) cells are depicted for uninoculated (**a**, **d**), 2+ CPE (**b**, **e**) and 4+ CPE (**c**, **f**) due to growth of poliovirus

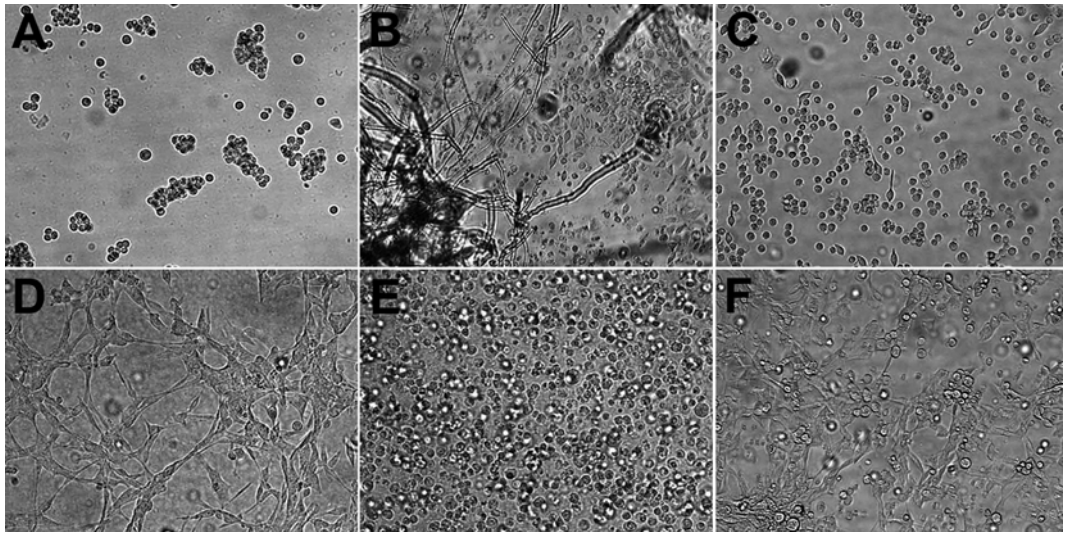

Fig. 2 Examples of non-enterovirus cytopathic effect in the L20B (panels **a**, **b** and **c**) and RD (panels **d**, **e**, and **f**) cell lines are depicted for toxicity (**a**, **d**), fungus (**b**), bacteria (**e**), and adenovirus (**c**, **f**)

affected: 1+ up to 25 %, 2+ up to 50 %, 3+ up to 75 %, and 4+ up to 100 %. Note the appearance of the CPE as to whether the cells are lysed, rounded, granular, non-adherent, etc. CPE may initially be observed as a focal point and should be left to develop until it covers more than 50 % of the cell monolayer (3+). A permanent record of the cell culture readings should be made either electronically or in a book.

8. If the tube cultures of both cell lines are lysed (4+) within 24 h of the primary inoculation, the specimen extract is toxic and, potentially, there was insufficient time for virus infection to be established. Discard the tubes and repeat the inoculation of the specimen extract diluted 1/10 with complete PBS to reduce the toxicity.
9. When CPE is observed over more than half of the cell monolayer (3+), passage the tube culture to a fresh monolayer of cells (*see* **Note 21**).
   (a) Lay the tubes horizontally in a plastic container with the orientation marker facing upwards so the media covers the remaining adherent cells. Place the container in a freezer at ≤−20 °C until the media is frozen so as to lyse any remaining intact cells and release virus particles.
   (b) Remove the container from the freezer and place the tubes upright in a rack leaving to thaw at ambient temperature. **Steps** (**a**) and (**b**) may be repeated to ensure complete lysis of the remaining intact cells, in particular if the CPE had not progressed beyond 75 % of the monolayer (4+).
   (c) Pellet the cellular debris by centrifuging at 1500×*g* for 5 min in a refrigerated bench centrifuge.
   (d) Transfer the supernatant to a labeled screw-capped storage vial.
   (e) Label new tube cultures with the unique code and the passage history: R_1L_1, R_2, etc.
   (f) Discard the media from the required number of new tube cultures, including a negative cell control tube, and replace with 2 ml maintenance media.
   (g) Inoculate the tube cultures with 0.2 ml of harvested supernatant from step (c).
   (h) Incubate tube cultures at 36 °C and observe daily under a microscope for CPE and record the reading.
10. Characteristic enterovirus CPE is observed as cells becoming rounded and refractile and may appear granular, leading to lysis and detachment as the CPE progresses (Fig. 1). Harvest the tube culture lysates displaying enterovirus-like CPE by following **steps 9** (**a**)–(**d**). Poliovirus does not have a characteristic CPE that can be distinguished from that caused by other enteroviruses. Further tests must be performed to confirm the presence of poliovirus including immunofluorescence (refer to Subheading 3.4), which can be used to quickly detect poliovirus in the cell lysate.
11. If no CPE is observed after 5–7 days incubation of the primary inoculation of specimen extract at **step 5**, perform a blind passage by harvesting the cell lysate and inoculating 0.2 ml on a

new monolayer of the same cell line as described at **step 9**. Observe the passaged cultures for a further 5–7 days (10–14 days total incubation) and if no CPE develops, discard the tubes and record the specimen as negative for poliovirus by cell culture.

12. Microorganisms other than enterovirus can cause CPE in tube cultures, in particular when stool specimen extracts are incubated (Fig. 2). Fungal contamination can be observed by the naked eye or microscopic observation of distinctive hyphae (Fig. 2b). Turbid culture media can be evidence of bacterial contamination that is observed microscopically as a haze and may create a barely perceptible movement in the media; the cells may display signs of degeneration (Fig. 2e). Discard the contaminated culture tubes and record the nature of the contamination. If primary inoculation tubes were contaminated after **step 5**, return to the original specimen extract, re-treat with chloroform and inoculate fresh culture tubes, recording this as a repeat primary inoculation; L_1, R_1, etc. Additional antibiotics may also be added to the culture media. If the contamination of a passaged culture occurred (L_2, R_1L_1, etc.), filter the primary inoculation through a 0.2 μm membrane using a syringe and collect the filtrate in a newly labeled tube. Inoculate 0.2 ml of the filtrate on a fresh cell monolayer and record this as a repeat passage. Other viruses such as adenovirus have a distinctive CPE that causes cell rounding and detachment usually without observation of concomitant lysis (Fig. 2c, f). A putative adenovirus CPE can be passaged from RD to RD cells but observation of adenovirus-like CPE in L20B is usually restricted to a single inoculation that will not recur when passaged to either the RD or L20B cell line. Specific testing (immunofluorescence, virus neutralization, PCR) is required to confirm the presence of adenovirus in the cell lysate.

3.5 Characterization of Poliovirus Isolates

A number of tests can be used to confirm the presence of poliovirus in tube cultures with an enterovirus-like CPE. The classic technique was antisera neutralization. Serotype-specific monoclonal and polyclonal antibodies raised against poliovirus are available commercially, as well as pooled antisera against the three serotypes. The harvested cell lysate, or isolate, is incubated with antiserum known to be capable of binding to the poliovirus virion, interfering with the process of cellular infection, and thus, preventing the development of viral CPE when subsequently incubated with a cell suspension. An isolate incubated in parallel with and without anti-poliovirus type 1 antiserum that does not exhibit CPE in the presence of antiserum, but does in its absence, is reported as poliovirus serotype 1. This technique has now been supplanted by RT-PCR methodology due to its rapid turnaround, capacity for high throughput and convenience, not being dependent upon the

immediate availability of sufficient cells. Oligonucleotide primers, fluorescent probes and thermal cycler conditions have been published to detect the individual poliovirus serotypes by RT-PCR and all three serotypes (pan-poliovirus). The following protocol describes an indirect immunofluorescence test to rapidly confirm the presence of poliovirus upon observation of enterovirus-like CPE in culture that, unlike antisera neutralization, does not require additional handling of cell lines. Commercial preparations of poliovirus serotype specific antibodies are available for this method but the use of a blend of antibodies to all three poliovirus serotypes will be described.

1. Upon observing enterovirus-like CPE in tube cultures but prior to harvesting the cell lysate by freeze-thawing (Subheading 3.4, **step 10**), pellet the cellular debris at 250×*g* for 10 min at ambient temperature (*see* **Note 22**). Remove the supernatant and store in a separate tube.
2. Add 250 μl of complete PBS with a transfer pipette and gently resuspend the cell pellet. Using the same pipette, transfer a single drop of suspension to each well of a three-well hydrophobic-coated microscope slide and let air-dry in a BSC. The remaining lysate may be stored at 4 °C pending the outcome of the immunofluorescence test or be harvested as described in Subheading 3.4 **step 9** (**a**)–(**d**).
3. Prepare three negative control microscope slides in the same manner as described in **steps 1** and **2** using an uninoculated tube culture.
4. Fill a microscope slide staining jar with cold acetone. Fix the cells to the slides by placing the slides in the jar for 10 min at 4 °C and air-dry.
5. During the incubation, dilute the concentrated mouse anti-poliovirus blend antibody preparation, stored at −20 °C, with PBS. Following the manufacturer's recommendation, prepare a range of dilutions to optimize the fluorescent staining; for example, 1:100, 1:200, and 1:400.
6. Add 15 μl of each dilution of anti-poliovirus antibody to just cover the fixed cellular material on a test and control slide.
7. Lay slides on a tray and place in a plastic container lined with paper towel moistened with water. Seal the humidified chamber with a lid and incubate at 37 °C for 30 min.
8. Remove the slides from the humidified chamber and rinse in a microscope staining jar filled with complete PBS for 5 min. Repeat the 5 min rinse with fresh complete PBS.
9. During the rinsing step, dilute the concentrated anti-mouse IgG-FITC labeled secondary antibody preparation, stored at −20 °C, with complete PBS. Follow the manufacturer's

recommendation or dilute the secondary antibody 1:100 with complete PBS.

10. Add 15 μl of diluted anti-mouse IgG-FITC labeled secondary antibody to just cover the fixed cellular material on each of the test and control slides.
11. Lay slides on a tray and place in a sealed humidified chamber and incubate at 37 °C for 30 min.
12. Remove the slides from the chamber and rinse in a microscope staining jar filled with complete PBS for 5 min. Repeat the 5 min rinse with fresh complete PBS.
13. Mount the slides with glycerol.
14. Observe the stained microscope slides at 10–100× using a fluorescence microscope with a filter suitable for FITC. A positive result for the putative poliovirus test slides should appear as a yellow-green fluorescence in the nucleus and/or cytoplasm of the cellular material. The negative control slides should be dull with only punctate or diffuse fluorescence and may have a red appearance if a rhodamine-red counter stain was included.

4 Notes

1. While short-term exposure of complete cell culture medium to fluorescent lighting during usage is acceptable, stocks should be stored in the dark to avoid production of toxic chemicals due to photoactivation of HEPES, riboflavin and tryptophan.
2. Batch-to-batch variation of serum can occur. An aliquot of FBS should be requested from the manufacturer for pretesting with the cell lines in routine usage. Acceptance of the batch of FBS can be based on cell morphology, growth rate, and sensitivity to poliovirus infection and other enteroviruses in parallel with the serum currently used. FBS must be heated at 56 °C for 30 min to inactivate complement proteins prior to addition to cell culture media. Gentle swirling of the serum at 5–10 min intervals during the inactivation will ensure even heat distribution. FBS should not be thawed more than twice and aliquots can be prepared after heat inactivation.
3. Cell sensitivity is measured by routinely testing the cell lines with standard poliovirus preparations of known titre for each serotype. WHO recommend testing is performed at least once per cell revival from liquid nitrogen [12].
4. Cell lines are usually shipped to laboratories in one or two 25 cm^2 flasks. The cells should be revived from liquid nitrogen one or two days before sending. On the day of shipment, the confluency of the cells is assessed and fresh media added to the

flask. The percentage of FBS in the media will depend upon the confluency of the cells; >50 % confluency use media with 2 % FBS, between 25 and 50 % confluency use 6 % FBS and for cells <25 % confluency use media with 10 % FBS, although it may be preferable to delay the shipment until the cells are more established. The culture media is added to the brim of the flask while in an upright position. Laboratory sealing film is wound tightly around the lid in a clockwise direction to prevent leakage and avoid loosening while in transit. Sufficient absorbent material should be included in the shipment package in case of leakage. Cell lines are shipped at ambient temperature and the courier should be informed that the contents are fragile and must avoid extreme temperatures but does not need to be refrigerated if there is a delay in transit.

5. The action of EDTA is to chelate divalent cations involved with cell attachment to other cells and the surface of the flask. Trypsin is a protease that cleaves amino acids involved with cell attachment and is inhibited by magnesium and chloride ions. Trypsin is often sourced from pig pancreases although recombinant forms are available.
6. With routine handling the period of incubation required to detach a particular cell line will be determined and may proceed in a single step. Some laboratories incubate the cells with trypsin–EDTA only until observing the initial rounding of the cells and prior to detachment. The trypsin–EDTA solution is removed from the flask in a BSC and returned to the incubator for a further period until cell detachment occurs. The authors have not noticed any untoward effects of leaving the trypsin–EDTA solution in the flask throughout the detachment procedure as it will become inactivated upon addition of the growth media at the next step.
7. Performing a cell count each time a cell line is passaged will ensure accurate and consistent results with all laboratory staff. However, sometimes it is sufficient to serial passage a cell line by performing a simple dilution or split ratio of the cell suspension. The growth pattern of a particular cell line is determined by routine handling as well as the dilution required to achieve a confluent cell monolayer in a specified number of days. For a split ratio of 1:5, 1 ml of cell suspension is diluted with 5 ml of growth media.
8. Trypan blue is a toluidine based dye that can traverse the membrane of nonviable cells but is excluded by intact cell membranes. Trypan blue is a hazardous chemical and suspected carcinogen that should be handled with gloves. Only discard Trypan blue waste or unused dye solution via a professional chemical waste disposal company.

9. Place the hemocytometer on a microscope stage and observe the grid pattern etched on each side of the chamber. Check the manufacturer's instructions for performing cell counts as the grid pattern and volume of the counting chamber can vary. The cell concentration is determined by counting viable (non-stained) cells in a specified number of squares on each side of the counting chamber. Nonviable cells that have taken up the Trypan blue dye are excluded from the count. A hand tally counter is useful to keep count. Only single cells should be counted, not clumps. If many clumps or aggregates of cells are observed the cell suspension requires further dispersal with a transfer pipette prior to dilution with Trypan blue. Individual cells that overlap the top and right hand side grid lines are included in the count but not those overlapping the bottom and left hand side grid lines.
10. The seeding concentration for ampoules stored in the cell bank can vary from 1 to 5×10^6 cells per ml depending on the intended usage and how quickly a confluent cell monolayer is needed after revival. Ampoules can be stored at 1×10^6 cells per ml if they are routinely revived into 25 cm^2 flasks whereas a 75 cm^2 flask requires a higher cell concentration at 5×10^6 cells per ml. The cell concentration in the cell bank should be higher than that for a normal passage to allow for loss of cell viability upon revival.
11. Liquid nitrogen is highly dangerous and should be handled with caution at all times. A SOP should be developed for training, routine work and emergency procedures. At atmospheric pressure the liquid is at –196 °C and can cause cold burns if splashed on exposed skin. Personal protective equipment including full-face mask, protective full-length gown and full-length gloves suitable for cryogenic work and covered shoes must be worn by all persons handling liquid nitrogen. As liquid nitrogen evaporates it reduces the oxygen concentration and asphyxiation can occur rapidly if storage containers are opened in enclosed spaces; 1 l of liquid nitrogen can expand to 168 l of nitrogen gas. For this reason liquid nitrogen containers must be stored in a well-ventilated area and a "buddy" assigned to keep watch on procedures outside of the store room to respond in an emergency. A personal oxygen meter can be attached to the protective gown of the lab worker handling liquid nitrogen to monitor the immediate area and sound an alarm if the oxygen concentration falls below a preset limit. In the event of a possible asphyxiation the buddy should not enter the liquid nitrogen store room unless wearing portable oxygen breathing apparatus.
12. Plastic cell culture tubes treated for adherent cell lines should be used. Tubes are approximately 16×125 mm with a screw capped lid that is kept closed during all incubations. Once

inoculated, tubes should not be opened to change the media of negative virus cultures due to the potential for contamination. Tubes with a built-in filter membrane in the lid are available for use with CO_2 incubators. Completely rounded culture tubes require a holder that fits on the microscope stage to assist viewing of the cell monolayer. Alternatively, tubes with a flattened side on the lower surface can be placed directly on the microscope stage. Glass tubes, while providing a clearer field when viewed under the microscope compared to plastic, are not recommended due to biosafety concerns if broken while containing live culture material. Cultures grown in 24 well plates are not recommended for poliovirus isolation due to the potential for contamination from adjacent wells.

13. The number of times a cell line has been passaged after revival from liquid nitrogen should be recorded with each passage. This can be indicated with a permanent marker on the cell culture flask or on a rack for tube cultures with a piece of removable tape labeled with the passage number of the cell line revived from liquid nitrogen followed by the number of times the cell line has been passaged since revival. For example, 20-7 indicates passage 20 is stored in liquid nitrogen and it has been passaged seven times out of a recommended maximum of 15 since revival.
14. The culture tubes need to remain stationery after seeding until the cell monolayer has formed. The rack holding the tubes should be tilted slightly backwards (approximately 5°) so the cells settle towards the end of the tube. The rack can be slanted by attaching a short piece of wooden rod, approximately 5 mm diameter, under the front surface of the rack. If the culture tubes were manufactured with an orientation indicator, turn the tubes in the rack so the indicator is at the top, otherwise indicate the orientation of the tube by drawing a line using a permanent marker. With the orientation indicator turned to the top, the cell monolayer will grow on the lower surface. Once the cell monolayer has formed in the tubes, the L20B cells remain in a stationery rack while the RD tubes are transferred to a tubeholder roller drum assembly that turns at approximately 0.2 rpm.
15. The waste container should be autoclavable and have a separate lid that can be secured prior to removal from the BSC. Line the container with a paper autoclave bag large enough to completely fill the container and be wrapped over the edge. Line the paper bag with a plastic bag of similar size as the paper bag. Place a large wad of absorbent cotton wool in the base of the container and spray the entire lining with disinfectant. Do not over fill with waste; a new container may need to be prepared if many specimens are processed at the same time. When the work procedure is completed, fold the plastic lining

inwards, followed by the paper bag lining, to cover the contents. Secure the lid, decontaminate the outer surface, and remove from the BSC. After autoclaving, carefully remove the paper bag as it may be soggy and dispose in a biological waste bin. Rinse the waste container and leave to dry.

16. As part of training for this procedure, it is recommended new staff weigh out 2 g of stool specimen to visually estimate the amount involved.
17. The purpose of chloroform is to extract lipid material, remove bacteria and fungi and to assist with disaggregation of non-enveloped virus particles. Note that if the specimen extract is to be also tested for enveloped viruses, chloroform treatment will denature the lipid membrane and an aliquot should be removed prior to the addition of chloroform.
18. The mechanical shaker must have a vigorous action that completely mixes the specimen extract either by vortex or horizontal agitation. The specimen extract may not be properly processed to fully separate the poliovirus particles from the organic matter if the mechanical shaker has a feeble mixing action.
19. It is important to record the passage history of virus cultures. Primary inoculation of a cell culture tube with specimen extract can be labeled as R_1 and L_1 on the RD and L20B cell line, respectively. Passage of R_1 to a fresh monolayer of RD cells would be indicated as R_2 and to L20B would be R_1L_1. Passage of L_1 to L20B would be indicated as L_2 and to RD would be L_1R_1.
20. Specimen extracts, especially of stool specimens, can be toxic when in direct contact with the cell monolayer. When inoculating culture tubes pipette the specimen extract down the inner face of the tube opposite the monolayer (same side as the orientation indicator) or directly into the culture media, cap the tube and gently swirl to mix and dilute the extract with the media.
21. At least two sequential incubations of cell monolayers are required for virus culture. The primary inoculation of the specimen extract will enable virus attachment, internalization, and replication to occur within cells sensitive to poliovirus infection but this can occur in competition with cell degeneration due to material present in specimen extracts that are toxic to the cells. Passage of the primary inoculation to a fresh monolayer will assist to identify that the CPE observed is due to poliovirus rather than toxicity; virus titre should increase when passaged to a fresh monolayer whereas toxic material will be diluted. The WHO algorithm for poliovirus isolation recommends passaging between the alternate cell lines for the rapid isolation of poliovirus rather than non-polio enteroviruses (NPEVs); a positive L20B isolate (L_1) is passaged to a fresh monolayer of RD cells and vice versa, R_1 to L20B. If R_1L_1 is

subsequently positive, a further passage back to the RD cell line is recommended as poliovirus usually grows to a higher titre in the RD cell line than L20B to conduct confirmatory tests. An additional passage of positive RD isolates (R_1) back to the RD cell line can help identify the isolation of NPEVs in the R_2 isolate. Since most NPEVs do not grow in the L20B cell line, the other passages (R_1L_1 and L_1R_1) will be negative. However, the L_1R_1 passage can be positive due to growth of an NPEV in the RD cell line from remnant NPEV particles present in the original stool extract inoculation of L20B culture media and carried over with the cell lysate. All positive virus cultures should be confirmed with specialized protocols used for putative poliovirus isolates that can also differentiate between wild and Sabin vaccine strains of poliovirus, known as intratypic differentiation.

22. The immunofluorescence test requires some cellular debris to remain for optimal sensitivity as poliovirus is more easily detected when attached to cellular membranes and fragments rather than floating in culture media. The CPE should be between 25 and 90 % to avoid false negatives due to either too little virus present or lack of cellular material, respectively.

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