

## APPENDIX I

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### CULTURE MEDIA, REAGENTS AND MOLECULAR TECHNIQUES

#### I. CELL CULTURE TECHNIQUES AND REAGENTS

##### A. Buffalo green monkey kidney, human epidermoid carcinoma and primary liver carcinoma cell line cultivation (Whitaker, 1972):

1. Five percent of Eagle's Minimum Essential Medium (MEM) (Highveld Biological, Lyndhurst, South Africa) was used as the growth medium for the cultivation of buffalo green monkey kidney (BGM), human epidermoid carcinoma (HEp-2) and primary liver carcinoma (PLC/PRF/5) cell lines (American Type Culture Collection, Virginia, United States of America [USA]).
2. The medium was replaced twice weekly. An yellow colour indicated the growth of cells and the production of metabolic substances due to acid excretion.
3. As soon as a monolayer of BGM, HEp-2 or PLC/PRF/5 cell lines was formed on the base of the 250 ml tissue culture flask (Cellstar, Greiner Labortechnik), the cells were passaged in a ratio of 1:2 to 1:6, depending on the thickness of the cell monolayer.
4. The medium in the flask was discarded and the walls of the flask were washed with 5 ml of phosphate-buffered saline (PBS) (Sigma Chemical Co., Louis, USA).
5. Three millilitres of trypsin EDTA (National Institute for Virology, South Africa) were added to the flask and incubated at 37°C for 1 min in a 5% CO<sub>2</sub> incubator (Galaxy CO<sub>2</sub> Incubator- Biotech, Northants, England).
6. The cells were detached from the bottom of the flask by lightly tapping the flask firmly against the hand.
7. An amount of 3 ml of a 10% MEM (Highveld Biological) was added to the flask and swirled to neutralise the effect of trypsin EDTA (National Institute for Virology, South Africa).
8. The whole suspension was transferred from the flask to a centrifuge tube (Corning Costar Corporation, Cambridge, Canada), which was centrifuged at 500 x g (BHG Roto-Uni II, Separation Scientific, South Africa) for 2 min in order to pellet the cells.

9. The supernatant was discarded and the pellet of cells was re-dissolved in 10% MEM (Highveld Biological).
10. After thorough mixing, 1 ml of the cell suspension was transferred into each flask containing 14 ml of the 5% MEM (Highveld Biological) that has been pre-heated to 37°C. Flasks were incubated at 37°C in a 5% CO<sub>2</sub> incubator (Galaxy CO<sub>2</sub> Incubator).

**B. Preparation of 5% MEM (Whitaker, 1972):**

1. Foetal calf serum (FCS) (Delta Bioproducts, Kempton Park, South Africa) was de-complemented in a water bath (Gallenkamp, England) at 56°C for 30 min.
2. Thirty millilitres of serum-free medium were abstracted from a 500 ml MEM bottle (Highveld Biological).
3. Twenty-five millilitres of filter sterilised foetal calf serum and 5 ml of sterile penicillin/streptomycin (pen/strep) fungizone mix (Whittaker M.A. Bioproducts, Maryland, USA) were added aseptically to the medium.

**C. Preparation of 10% MEM (Whitaker, 1972):**

1. Foetal calf serum (FCS) (Delta Bioproducts) was de-complemented in a water bath (Gallenkamp, England) at 56°C for 30 min.
2. Fifty-five millilitres of serum-free medium were abstracted from a 500 ml MEM bottle (Highveld Biological).
3. Fifty millilitres of filter sterilised foetal calf serum and 5 ml of sterile pen/strep fungizone mix (Whittaker) were added aseptically to the medium.

**D. Determination of total BGM, HEp-2 and PLC/PRF/5 cell counts (Bird and Forrester, 1981):**

1. The total number of viable cells of a monolayer of BGM (HEp-2 and PLC/PRF/5 cells) formed on the base of the flask (250 ml) was determined.
2. The medium in the flask was discarded and the walls of the flask were washed with 5 ml phosphate-buffered saline (PBS) (Sigma).
3. Trypsin EDTA (National Institute for Virology, South Africa) (3 ml) was added to the flask. The flask was incubated at 37°C for 1 min.
4. The cells were detached from the bottom of the flask by lightly tapping the flask firmly against the hand.

5. Three millilitres of a 10% MEM (Highveld Biological) were added to the flask and swirled to neutralise the effect of trypsin EDTA.
6. The suspension was transferred from the flask to a centrifuge tube (Corning Costar), which was centrifuged at 500 x g (BHG Roto-Uni II) for 2 min to pellet the cells.
7. The supernatant was discarded and the pellet of cells was re-dissolved by mixing thoroughly in a serum-free media, with a volume (generally,  $V_{\text{initial}} = 10$  ml) dependent on the thickness of the monolayer.
8. In a separate centrifuge tube, 500  $\mu$ l of 0.4% trypan blue stain (Sigma) were added. This cell counting method was based on the fact that viable cells (with intact membranes) do not take up the trypan blue stain, whereas non-viable cells do.
9. An equal amount of the cell suspension (500  $\mu$ l) (dilution factor of 2) was added to the trypan blue and mixed thoroughly to receive an even suspension without excessive clumping. The trypan blue-cell suspension was stained for 5 min.
10. A cover-slip was placed on one of the two counting chambers of a Neubauer hemacytometer (Superior, Germany). This counting chamber had four identical ruled squares, each measuring 1 by 1 mm. The space between the cover-slip and the ruled squares (with surface areas of 1 mm<sup>2</sup>) was 0.1 mm. Therefore, the volume of one ruled square was 0.1 mm<sup>3</sup>, or 10<sup>-4</sup> cm<sup>3</sup>. Using a Pasteur pipette, a small amount of the trypan blue-cell suspension (10  $\mu$ l) was transferred to the counting chamber by carefully touching the edge of the cover-slip with the pipette tip and allowing the chamber to fill by capillary action. The chamber was not allowed to overflow or underfill.
11. The counting chamber was placed and examined under a light microscope. The low-power objective was focused on the ruled squares and all of the cells in the four 1 mm<sup>2</sup> corner squares were counted. Cells lying outside the borders of the squares were not counted.
12. The total number of viable cells in the 4 squares was divided by 4 to determine the mean count per square. This represented the number of cells per 0.1 mm<sup>3</sup>. This number is multiplied by 10 000 to determine the number of cells per cubic centimetre. Since 1 cm<sup>3</sup> is equivalent to 1 ml, the cell number can be expressed per millilitre. The final number is adjusted by the appropriate dilution factor.

**Determination of the total number of viable cells (Bird and Forrester, 1981):**

**Cells per ml** = the average count per square  $\times 10^4$

**Total cells** = cells per ml  $\times$  the dilution factor  $\times$  the original volume of fluid from which cell sample was removed.

**Example:**

$$C_{\text{initial}} = 24 \times 10^4 \text{ cells.ml}^{-1}$$

$$C_{\text{final}} = 2 \times 10^5 \text{ cells.ml}^{-1}$$

$$V_{\text{initial}} = 10 \text{ ml}$$

$$2 \times C_{\text{initial}} \times V_{\text{initial}} = C_{\text{final}} \times V_{\text{final}}$$

$$2 \times (24 \times 10^4 \text{ cells.ml}^{-1}) \times 10 \text{ ml} = (2 \times 10^5 \text{ cells.ml}^{-1}) \times V_{\text{final}}$$

$$V_{\text{final}} = (4.8 \times 10^6 \text{ cells.ml}^{-1}) / (2 \times 10^5 \text{ cells.ml}^{-1})$$

$$V_{\text{final}} = 24 \text{ ml}$$

13. The pellet of cells was dissolved in serum-free media and centrifuged at 500  $\times$  g (BHG Roto-Uni II) for 2 min to pellet the cells.
14. The supernatant was discarded and the cell pellet was re-dissolved in the necessary volume ( $V_{\text{final}}$ ) of 5% MEM growth medium.

**E. Freezing of BGM, HEp-2 and PLC/PRF/5 cell cultures for storage (Whittaker, 1972):**

Add the following to prepare the freeze medium:

1. Twenty-five millilitres of filter sterilised double-strength Eagle's Minimum Essential Medium (Highveld Biological).
2. Twenty millilitres of filter sterilised foetal bovine serum (Delta Bioproducts).
3. Five millilitres of filter sterilised glycerol (Sigma).
4. Sterile pen/strep fungizone mix (0.5 ml) (Whittaker).
5. Mix together.

**Procedure:**

1. Cells were trypsinised and neutralised with 10% MEM (Highveld Biological) medium.
2. The cell suspension was centrifuged at 500  $\times$  g (BHG Roto-Uni II) to pellet cells.
3. The supernatant was removed and cells were re-suspended in 1 ml of freeze-medium.
4. The cell suspension was carried over to a sterile cryogenic vial (Corning Costar) and frozen at 4°C for 2 h.

5. After 2 h, the cell suspension was frozen at  $-20^{\circ}\text{C}$  for 24 h and finally the cell suspension was stored at  $-70^{\circ}\text{C}$  for future analysis.

## **II. RECOVERY OF VIRUSES FROM SEWAGE AND RIVER WATER SAMPLES**

### **A. Decontamination of the sewage and river water samples (Minor, 1985):**

1. Sewage and river water samples were decontaminated using chloroform (Merck, Darmstadt, Germany). Ten millilitres of chloroform were added to 50 ml of sewage/river water samples and mixed for 30 min in a shaking incubator (Labcon, Labotec, South Africa) at 200 rpm ( $\pm 25^{\circ}\text{C}$ ).
2. The samples were centrifuged at  $4\,500 \times g$  (Sorvall Super T 21, Wilmington, USA) at  $4^{\circ}\text{C}$  in order to separate the supernatant from the chloroform.

### **B. Polyethylene glycol/sodium chloride precipitation method (Minor, 1985):**

1. A mixture consisting of 14.0 g polyethylene glycol (PEG) (PEG 6000, Merck) and 1.17 g sodium chloride (NaCl) (Sigma) was prepared and this mixture was dissolved into 100 ml of sewage/river water sample.
2. After settling down at  $4^{\circ}\text{C}$  for 24 h, each sample was divided into two 50 ml centrifuge tubes (Corning Costar) and each sample tube was centrifuged at  $2\,500 \times g$  (Sorvall Super T 21) for 30 min at  $4^{\circ}\text{C}$ .
3. The supernatant was discarded and 10 ml of PBS (Sigma) were added into each centrifuge tube. The two tubes of each sample were added together and mixed well.
4. The sonicator (Soniprep 150, MSE) was cleaned with 70% ethanol (Merck) before use and the samples were sonicated for 20 s in order to break loose any pre-formed virus clumps.
5. The samples were centrifuged at  $600 \times g$  (BHG Roto-Uni II) for 10 min at  $4^{\circ}\text{C}$  before being added to bottles containing nystatin and penicillin/streptomycin neomycin mix (150  $\mu\text{l}$  of each antibiotic) (Whittaker).
6. Finally, the sewage and river water samples were stored at  $-20^{\circ}\text{C}$  for further analysis.

### III. ENTEROVIRUS DETECTION

#### A. **Plaque assay for the detection of plaque forming enteroviruses (Manor *et al.*, 1999):**

1. The BGM, HEp-2 and PLC/PRF/5 cells were seeded in 92 mm Nunclon tissue culture plates (Nalge Nunc, Denmark) at a concentration of  $2 \times 10^5$  cells.ml<sup>-1</sup> and incubated at 37°C in a 5% CO<sub>2</sub> incubator (Galaxy CO<sub>2</sub> Incubator).
2. After 48 h of incubation, the medium was discarded aseptically and a confluent BGM, HEp-2 or PLC/PRF/5 cell monolayer was observed at the bottom of each tissue culture plate.
3. The cells were starved by adding 2 ml of a medium consisting of MEM and 1% pen/strep fungizone mix on the side of each tissue culture plate.
4. The infectious plates were incubated for 1 h at 37°C. After 1 h, the MEM + 1% pen/strep fungizone medium was pulled off.
5. The cells were infected with 1 ml of the PEG sample (sewage and river water) and incubated for 1 h 30 min at 37°C with rotation of the flask every 10-15 min.
6. Overlay medium stock was prepared using filter sterilised double-strength Eagle's MEM, consisting of 96% MEM and 4% foetal bovine serum. A 2% Sea Kem ME Agarose solution (FMC Bioproducts, ME, USA), consisting of 2 g agar in 100 ml of PBS (pH 7.2) (Sigma), was prepared separately and autoclaved at 121°C for 15 min. Equal amounts of the double-strength MEM and the agar were mixed at 50°C to give a final concentration of 1% agar.
7. Each tissue culture plate received 20 ml of the overlay medium, which was poured aseptically on the side of each plate without disrupting the pre-formed BGM, HEp-2 or PLC/PRF/5 cell monolayers.
8. The agarose in the tissue culture plates was allowed to solidify for 10-20 min at 22°C.
9. The tissue culture plates were incubated at 37°C and plaques appeared within 5 - 7 days.
10. Plaques were picked up from the plates and the virus was allowed to replicate in 50 ml tissue culture flasks (Cellstar, Greiner Labortechnik) containing pre-formed BGM, HEp-2 and PLC/PRF/5 cell monolayers.
11. The concentrated virus was extracted from each tissue culture flask and stored at -20°C for further analysis.

**B. Extraction of the ribonucleic acid from sewage and river water samples (RNeasy, Qiagen, Hilden, Germany):**

1. The ribonucleic acid (RNA) was extracted by means of a RNeasy Viral RNA extraction kit (RNeasy, Qiagen, Hilden, Germany).
2. A total of 1.5 ml of virus infected cells were centrifuged at 300 x g (Eppendorf Centrifuge 5402D, Hamburg, Germany) for 5 min ( $\pm 25^{\circ}\text{C}$ ).
3. The supernatant was removed carefully by aspiration.
4. The cells were disrupted by the addition of buffer RLT (350  $\mu\text{l}$  per reaction). A  $\beta$ -mercaptoethanol (10  $\mu\text{l}$ ) was mixed with 1 ml of buffer RLT before use. The cell pellets were dissolved thoroughly by inverting each centrifuge tube.
5. Each lysate was pipetted directly onto QIAshredder spin columns placed in 2 ml collection tubes and the lysates were centrifuged for 2 min at a maximum speed (Eppendorf Centrifuge 5402D) at  $\pm 25^{\circ}\text{C}$ .
6. A volume of 350  $\mu\text{l}$  of 70% ethanol per reaction was added to each homogenised lysate and mixed well by pipetting.
7. Up to 700  $\mu\text{l}$  of each sample were added to RNeasy mini columns placed in 2 ml collection tubes (supplied) and each sample was centrifuged for 15 s at 8 000 x g (Eppendorf Centrifuge 5402D) at  $\pm 25^{\circ}\text{C}$ . The flow-through was discarded.
8. A buffer RW1 (350  $\mu\text{l}$  per reaction) was pipetted into each spin column and the spin columns were centrifuged for 15 s at 8 000 x g (Eppendorf Centrifuge 5402D) at  $\pm 25^{\circ}\text{C}$ . The flow-through was discarded and the collection tubes were re-used.
9. DNase I (10  $\mu\text{l}$ ) stock solution was added to 70  $\mu\text{l}$  of Buffer RDD and this solution was mixed gently by inverting each tube.
10. The DNase I incubation mix (80  $\mu\text{l}$  per reaction) was pipetted directly onto each spin-column membrane and the columns were placed on the benchtop ( $20^{\circ}\text{C}$  to  $30^{\circ}\text{C}$ ) for 15 min.
11. After 15 min, buffer RW1 (350  $\mu\text{l}$  per reaction) was pipetted into each spin column and the spin columns were centrifuged for 15 s at 8 000 x g (Eppendorf Centrifuge 5402D) at  $\pm 25^{\circ}\text{C}$ . The flow-through and collection tubes were discarded.
12. Each spin column was placed in a new 2 ml collection tube (supplied). Buffer RPE (500  $\mu\text{l}$  per reaction) was pipetted into each spin column and the spin columns were

centrifuged for 15 s at 8 000 x g (Eppendorf Centrifuge 5402D) at  $\pm 25^{\circ}\text{C}$ . The flow-through was discarded and each collection tube was re-used.

13. Buffer RPE (500  $\mu\text{l}$  per reaction) was pipetted into each spin column and the columns were centrifuged for 2 min at a maximum speed (Eppendorf Centrifuge 5402D) at  $\pm 25^{\circ}\text{C}$  to dry the silica-gel membranes, thus ensuring that no ethanol is carried over during elution.
14. Each spin column was transferred to new 1.5 ml collection tubes (supplied) and 55  $\mu\text{l}$  of RNase-free water were pipetted directly onto the silica-gel membranes. The columns were centrifuged for 1 min at 8 000 x g (Eppendorf Centrifuge 5402D) at  $\pm 25^{\circ}\text{C}$  in order to elute the RNA.
15. The extracted RNA was stored at  $-70^{\circ}\text{C}$  for further analysis.

**C. TRIzol method for the extraction of viral RNA from stool samples (Center for Pediatric Research, EVMS/CHKD, Virginia, USA):**

1. In the homogenisation and clarification of the stool specimens, 300  $\mu\text{l}$  of 10 - 50% faecal suspension was mixed with an equal volume of freon (Sigma).
2. The samples were vortexed for 30 s at  $\pm 25^{\circ}\text{C}$ .
3. The samples were centrifuged at 12 000 x g (Eppendorf Centrifuge 5402D) for 5 min at room temperature ( $\pm 25^{\circ}\text{C}$ ) and 140  $\mu\text{l}$  of each supernatant were withdrawn for RNA extraction.
4. The clarified stool suspensions (140  $\mu\text{l}$  per reaction) were mixed with 500  $\mu\text{l}$  of TRIzol (Invitrogen Life Techno, Paisley, Scotland). The mixtures were incubated at room temperature ( $\pm 25^{\circ}\text{C}$ ) for 5 min to permit complete dissociation of the nucleoprotein complex.
5. Pure chloroform (100  $\mu\text{l}$  per reaction) (Sigma) was added to 500  $\mu\text{l}$  of TRIzol and mixed vigorously by hand for 15 s. The mixtures were incubated for 3 min at room temperature ( $\pm 25^{\circ}\text{C}$ ). The samples were centrifuged at 12 000 x g (Eppendorf Centrifuge 5402D) for 15 min at  $4^{\circ}\text{C}$ .
6. In separate 1.5 ml eppendorf tubes (Eppendorf), 30  $\mu\text{l}$  of 3 M sodium acetate (pH 5.2) (Merck) per reaction were added to 600  $\mu\text{l}$  of 100% ethanol (Merck). After 15 min of centrifugation, the aqueous phase (300  $\mu\text{l}$ ) was transferred to each of the 1.5 ml eppendorf tubes.



7. The samples were stored at -20°C overnight.
8. After 24 h, the samples were centrifuged at 12 000 x g (Eppendorf Centrifuge 5402D) for 15 min at 4°C.
9. Each supernatant was discarded and the RNA pellets were washed with 300 µl of 70% ethanol (Merck) per reaction.
10. The samples were centrifuged at 12 000 x g (Eppendorf Centrifuge 5402D) for 5 min at 4°C. Each supernatant was discarded and the RNA pellets were air-dried from 5 to 10 min.
11. Each RNA pellet was dissolved in 35 µl of RNase free water (DEPC-water, Promega Corp., Madison, USA) and the samples were incubated for 10 min at 42°C in the hybridisation oven (Techne Hybridiser HB-1D, Techne, Cambridge, United Kingdom). After 10 min, the samples were centrifuged briefly at 8 000 x g (Eppendorf Centrifuge 5402D) at ±25°C and stored at -70°C for further analysis.

**D. Reverse transcription polymerase chain reaction for the detection of enteroviruses (Gow *et al.*, 1991):**

1. A reverse transcription polymerase chain reaction (RT-PCR) was carried out using a Promega Access RT-PCR kit.
2. Optimised final concentrations in a total volume of 50 µl included: AMV/*Tfl* reaction buffer (1x), 1.5 mM MgSO<sub>4</sub>, dNTP Mix (final concentration of 0.2 mM), 50 pmol each of primers EP1 and EP4 (Sigma-Genosys Ltd., Pampisford, Cambridgeshire, United Kingdom), 5 U each of AMV Reverse Transcriptase and *Tfl* DNA Polymerase.

**PCR conditions applied:**

48°C for 45 min, reverse transcription

94°C for 1 min, DNA denaturation  
56°C for 1 min, primer annealing  
72°C for 1 min, primer extension

} 30 cycles

72°C for 10 min, final extension

**Preparation of master mix cocktails:**

Reagents	Master mix per reaction
Nuclease-Free H <sub>2</sub> O	22.00 µl
AMV/ <i>Tfl</i> 1x Reaction Buffer	10.00 µl
DNTP Mix, 10 mM (10 mM each of dATP, dCTP, dGTP, dTTP)	1.00 µl
EP1 (50 pmol)	0.50 µl
EP4 (50 pmol)	0.50 µl
MgSO <sub>4</sub> , 25 mM	4.00 µl
AMV Reverse Transcriptase, 5 units.µl <sup>-1</sup> (0.1 M Potassium phosphate pH 7.2, 0.2% Triton-X-100, 2 mM DTT, 50% Glycerol)	1.00 µl
<i>Tfl</i> DNA Polymerase, 5 units.µl <sup>-1</sup> (50% Glycerol, 20 mM Tris HCl pH 8.0, 1 mM DTT, 100 mM KCl, 0.1 mM EDTA, 0.5% Nonidet P40, 0.5% Tween 20)	1.00 µl
<b>Total</b>	<b>40.00 µl</b>

3. In total, 40 µl of master mix cocktails were dispensed into 0.5 ml eppendorf tubes (Eppendorf).
4. Two drops of mineral oil (Promega Corp.) were added into each eppendorf tube to avoid evaporation of the PCR product.
5. Ten microlitres of extracted RNA were added into each eppendorf tube containing the PCR cocktails in an ultraviolet (UV) cabinet, to give a final volume of 50 µl per reaction.
6. The amplification (30 cycles) was performed in a Hybaid Thermocycler (Hybaid OmniGene Thermocycler, United Kingdom).
7. Enteroviruses displayed a 408 bp product.

**E. Nested PCR amplification (Kuan, 1997):**

1. A second PCR run was undertaken, in which 1 µl of the amplified RT-PCR product was added to 49 µl of previously prepared PCR mixture (Promega Corp.).
2. The PCR mixture contained the following: 1x PCR buffer (10 mM Tris-HCl, pH 9; 50 mM KCl; 0.1% Triton X-100), MgCl<sub>2</sub> (final concentration of 1.5 mM), dNTP mix (final concentration of 0.2 mM), 50 pmol each of primers E1 and E2 (Sigma-Genosys), and 1.5 U of *Taq* DNA polymerase (Promega Corp.).

**Nested PCR conditions applied:**

94°C for 3 min, DNA denaturation  
 94°C for 1 min, DNA denaturation  
 45°C for 1 min, primer annealing  
 72°C for 1 min, primer extension  
 72°C for 10 min, final extension

} 30 cycles

**Preparation of master mix cocktails:**

Reagents	Master mix per reaction
Nuclease-Free H <sub>2</sub> O	37.50 µl
1x PCR buffer	5.00 µl
MgCl <sub>2</sub>	4.00 µl
dNTP Mix, 10 mM (10 mM each of dATP, dCTP, dGTP, dTTP)	1.00 µl
E1 (50 pmol)	0.50 µl
E2 (10 pmol)	0.50 µl
<i>Taq</i> DNA Polymerase, 5 units.µl <sup>-1</sup> (50% Glycerol, 20 mM Tris HCl pH 8.0, 1 mM DTT, 100 mM KCl, 0.1 mM EDTA, 0.5% Nonidet P40, 0.5% Tween 20)	0.50 µl
<b>Total</b>	<b>49.00 µl</b>

3. In total, 49 µl of master mix cocktails were dispensed into 0.5 ml eppendorf tubes (Eppendorf).
4. One microlitre of each PCR product was added into each eppendorf tube containing the pre-formed master mix cocktails in an UV cabinet, to give a final volume of 50 µl per reaction.
5. After 30 cycles, 20 µl of each nested PCR product were subjected to agarose (2%) (Seakem LE agarose) gel electrophoresis (Midicell Primo Gel Apparatus).
6. Enteroviruses displayed a 297 bp product.

**F. Reverse transcription multiplex PCR to distinguish poliovirus from non-polio enteroviruses (Egger *et al.*, 1995):**

1. Primers specific for either enterovirus or poliovirus were combined in a reverse transcription multiplex PCR (RT-multiplex PCR) (Promega Access RT-PCR system).
2. Optimised final concentrations in a total volume of 50 µl were: AMV/*Tfl* reaction buffer (1x), 2.0 mM MgSO<sub>4</sub>, dNTP Mix (final concentration of 0.2 mM), 25 pmol each of primers E1, E2, Po1, Po2, Po3 and Po4 (Sigma-Genosys), 5 U each of AMV Reverse Transcriptase and *Tfl* DNA Polymerase (Promega Corp.).

**PCR conditions applied:**

48°C for 45 min, reverse transcription  
 94°C for 1 min, DNA denaturation  
 45°C for 1.5 min, primer annealing  
 72°C for 1 min, primer extension } 30 cycles  
 72°C for 10 min, final extension

**Preparation of master mix cocktails:**

Reagents	Master mix per reaction
Nuclease-Free H <sub>2</sub> O	22.50 µl
AMV/ <i>Tfl</i> 1x Reaction Buffer	10.00 µl
dNTP Mix, 10 mM (10 mM each of dATP, dCTP, dGTP, dTTP)	1.00 µl
E1 (25 pmol)	0.25 µl
E2 (25 pmol)	0.25 µl
Po1 (25 pmol)	0.25 µl
Po2 (25 pmol)	0.25 µl
Po3 (25 pmol)	0.25 µl
Po4 (25 pmol)	0.25 µl
MgSO <sub>4</sub> , 25 mM	3.00 µl
AMV Reverse Transcriptase, 5 units.µl <sup>-1</sup> (0.1 M Potassium phosphate pH 7.2, 0.2% Triton-X-100, 2 mM DTT, 50% Glycerol)	1.00 µl
<i>Tfl</i> DNA Polymerase, 5 units.µl <sup>-1</sup> (50% Glycerol, 20 mM Tris HCl pH 8.0, 1 mM DTT, 100 mM KCl, 0.1 mM EDTA, 0.5% Nonidet P40, 0.5% Tween 20)	1.00 µl
<b>Total</b>	<b>40.00 µl</b>

3. In total, 40 µl of master mix cocktails were dispensed into 0.5 ml eppendorf tubes (Eppendorf).
4. Two drops of mineral oil (Promega Corp.) were added into each eppendorf tube to avoid evaporation of the PCR product.
5. Ten microlitres of extracted RNA were added into each eppendorf tube containing the PCR cocktails in an UV cabinet, to give a final volume of 50 µl per reaction.
6. The amplification was performed in a Hybaid Thermocycler (Hybaid OmniGene Thermocycler).
7. After 30 cycles, 20 µl of each PCR product were subjected to agarose (2%) (Seakem LE agarose) gel electrophoresis (Midicell Primo Gel Apparatus).
8. Poliovirus vaccine strains displayed base pair bands at 193 bp, 297 bp, 565 bp and in several cases at 1 000 bp, whereas non-polio enteroviruses showed the enterovirus-specific 297 bp band.

**G. Sabin specific RT-triplex PCR to distinguish between the Sabin poliovirus types 1 to 3 (Yang *et al.*, 1991; Yang *et al.*, 1992):**

1. Three sets of primers specific for Sabin poliovirus vaccine strains were combined in a RT-triplex PCR to confirm the isolated polioviruses as OPV strains (Promega Corp.).
2. A 50 µl reaction volume containing the following was prepared: AMV/*Tfl* Reaction Buffer (1x), dNTP Mix (final concentration of 0.2 mM), 25 pmol each of primers S1-1, S1-2, S2-1, S2-2, S3-1a, S3-2 (Sigma-Genosys), 1.5 mM of MgSO<sub>4</sub>, 5 U each of AMV Reverse Transcriptase and *Tfl* DNA Polymerase (Promega Corp.).
3. The amplification was performed in 30 cycles in a Mini Thermocycler (MJ Research, Inc., Watertown, USA) or in a Hybaid Thermocycler (Hybaid OmniGene Thermocycler).

**PCR conditions applied:**

42°C for 45 min, reverse transcription

95°C for 30 s, DNA denaturation	} 30 cycles
56°C for 45 s, primer annealing	
72°C for 1 min, primer extension	

72°C for 10 min, final extension

**Preparation of master mix cocktails:**

Reagents	Master mix per reaction
Nuclease-Free H <sub>2</sub> O	22.50 µl
AMV/ <i>Tfl</i> 1x Reaction Buffer	10.00 µl
dNTP Mix, 10 mM (10 mM each of dATP, dCTP, dGTP, dTTP)	1.00 µl
S1-1 (25 pmol)	0.25 µl
S1-2 (25 pmol)	0.25 µl
S2-1 (25 pmol)	0.25 µl
S2-2 (25 pmol)	0.25 µl
S3-1a (25 pmol)	0.25 µl
S3-2 (25 pmol)	0.25 µl
MgSO <sub>4</sub> , 25 mM	3.00 µl
AMV Reverse Transcriptase, 5 units.µl <sup>-1</sup> (0.1 M Potassium phosphate pH 7.2, 0.2% Triton-X-100, 2 mM DTT, 50% Glycerol)	1.00 µl
<i>Tfl</i> DNA Polymerase, 5 units.µl <sup>-1</sup> (50% Glycerol, 20 mM Tris HCl pH 8.0, 1 mM DTT, 100 mM KCl, 0.1 mM EDTA, 0.5% Nonidet P40, 0.5% Tween 20)	1.00 µl
<b>Total</b>	<b>40.00 µl</b>

4. In total, 40  $\mu\text{l}$  of master mix cocktails were dispensed into 0.5 ml eppendorf tubes (Eppendorf).
5. Two drops of mineral oil (Promega Corp.) were added into each eppendorf tube to avoid evaporation of the PCR product.
6. Ten microlitres of extracted RNA were added into each eppendorf tube containing the PCR cocktails in an UV cabinet, to give a final volume of 50  $\mu\text{l}$  per reaction.
7. The amplification was performed in 30 cycles in a Mini Thermocycler (MJ Research) or in a Hybaid Thermocycler (Hybaid OmniGene Thermocycler).
8. The amplified products (20  $\mu\text{l}$ ) were separated with 7% polyacrylamide (BioRad, Hercules, California, USA) gel electrophoresis using a Hoefer electrophoresis unit (SE 600 Electrophoresis Unit, Hoefer, Scientific Instruments, San Francisco, California, USA).
9. Sabin poliovirus type 1 showed 97 bp band, Sabin poliovirus type 2 showed 71 bp band and Sabin poliovirus type 3 displayed 54 bp band.

**H. Restriction enzyme analysis (Kämmerer *et al.*, 1994; Kuan, 1997):**

1. Non-polio enteroviruses were typed using three restriction enzymes (*Sty* I, *Bgl* I and *Xmn* I) (Promega Corp.).
2. Aliquots of 10  $\mu\text{l}$  of the RT-multiplex PCR products were incubated with 10 U of each of the restriction enzymes in 30  $\mu\text{l}$  reaction volumes with the buffers recommended by the manufacturer (Promega Corp.).

**Preparation of master mix cocktails:**

Reagents	Master mix per reaction
<i>Sty</i> I (10 u. $\mu\text{l}^{-1}$ ), <i>Bgl</i> I (10 u. $\mu\text{l}^{-1}$ ), <i>Xmn</i> I (10 u. $\mu\text{l}^{-1}$ )	1.0 $\mu\text{l}$
Restriction enzyme 10xBuffer F, RE 10xBuffer D, RE 10xBuffer	3.0 $\mu\text{l}$
Acetylated BSA, 10 $\mu\text{g}.\mu\text{l}^{-1}$	0.3 $\mu\text{l}$
sterile, de-ionised H <sub>2</sub> O	15.7 $\mu\text{l}$
<b>Total</b>	<b>20.0 <math>\mu\text{l}</math></b>

3. In total, 20  $\mu\text{l}$  of master mix cocktails were dispensed into 0.5 ml eppendorf tubes (Eppendorf).
4. Ten microlitres of each RT-multiplex PCR product (obtained from method F) were added into each eppendorf tube containing the pre-formed master mix cocktails in an UV cabinet, to give a final volume of 30  $\mu\text{l}$  per reaction.

5. The samples were incubated at 37°C for 3 h in a Hybaid Thermocycler (Hybaid OmniGene Thermocycler) and were analysed using 7% polyacrylamide (BioRad) gel electrophoresis (Hofer, SE 600 Electrophoresis Unit).

**Fragments resulting from digestion by *Sty* I, *Bgl* I and *Xmn* I restriction enzymes of 297 bp amplified enteroviruses (Kämmerer *et al.*, 1994; Kuan, 1997)**

Restriction enzyme	Prototype enteroviruses	DNA fragment size (bp)
<i>Sty</i> I	ECV4, ECV9, ECV11, ECV20, PV3, CAV2, CAV3, CAV5, CAV7, CBV4	297
	ECV6, ECV19, PV1, CAV6, CBV2, CBV3	226 + 71
	ECV7, PV2, CBV1	197 + 100
	CBV2, CBV3, CBV6	212 + 75 + 10
	CBV5	112 + 102 + 83
<i>Bgl</i> I	ECV7, ECV9, ECV11, ECV20, PV2, PV3, CBV1, CBV3, CAV1, CAV5, CAV6, CAV7	297
	ECV4, ECV6, ECV14, ECV19, PV1, CAV2, CAV3, CBV2, CBV4, CBV5	217 + 80
	CBV6	196 + 80 + 21
<i>Xmn</i> I	ECV7, ECV11, PV2, PV3, CAV1, CBV3	297
	ECV4, ECV6, ECV9, ECV14, ECV20, PV1, CAV2, CAV3, CAV5, CAV6, CA7, CBV1, CBV2	236 + 61

**I. Preparation of 7% polyacrylamide gel**

1. In total, 7 ml of acrylamide (40%) (BioRad) were mixed with 29 ml of sterile distilled water.
2. Four millilitres of 10 x TAE (Amresco, Solon, Ohio, USA), 250 µl of ammonium persulphate (AMPS) (Amresco) and 50 µl of temed (Sigma) were added to the above solution.
3. A spacer mate (Hofer) and two spacers were placed on a glass plate (16 cm x 18 cm x 0.3 cm). A second glass plate was placed on top and the glass plates were tightened with clamps.
4. The spacer mate was removed and the gel was poured between the two glass plates.
5. After the gel solidified, the glass plates were placed in a Hofer electrophoresis unit. The gel was run at 120 Volts at 6°C using a BioRad power supply (BioRad, 500/200 Power Supply).

#### IV. NUCLEOTIDE SEQUENCE ANALYSIS

##### A. A RT-PCR for the 5'untranslated region of poliovirus vaccine strains (Divizia *et al.*, 1999; Guillot *et al.*, 2000):

1. The RT-PCR was performed as described by Divizia *et al.* (1999) and Guillot *et al.* (2000) with a few modifications.
2. The primers used for the 5'untranslated region (5'UTR) were as follows: UG52 (nt 160 to 180) and UC53 (nt 599 to 580) (Sigma-Genosys).
3. Optimised final concentrations in a total volume of 50  $\mu$ l were: AMV/*Tfl* Reaction Buffer (1x), 2.0 mM MgSO<sub>4</sub>, dNTP Mix (final concentration of 0.2 mM), 10 pmol each of primers UG52 and UC53 (Sigma-Genosys), 5 U each of AMV Reverse Transcriptase and *Tfl* DNA Polymerase (Promega Corp.).
4. The amplification was performed in 30 cycles in a Mini Thermocycler (MJ Research).

##### **PCR conditions applied:**

42°C for 45 min, reverse transcription  
 95°C for 30 s, DNA denaturation  
 45°C for 45 s, primer annealing  
 72°C for 1 min, primer extension  
 72°C for 10 min, final extension

} 30 cycles

##### **Preparation of master mix cocktails:**

Reagents	Master mix per reaction
Nuclease-Free H <sub>2</sub> O	28.80 $\mu$ l
AMV/ <i>Tfl</i> 1x Reaction Buffer	10.00 $\mu$ l
dNTP Mix, 10 mM (10 mM each of dATP, dCTP, dGTP, dTTP)	1.00 $\mu$ l
UG52 (10 pmol)	0.10 $\mu$ l
UC53 (10 pmol)	0.10 $\mu$ l
MgSO <sub>4</sub> , 25 mM	3.00 $\mu$ l
AMV Reverse Transcriptase, 5 units. $\mu$ l <sup>-1</sup> (0.1 M Potassium phosphate pH 7.2, 0.2% Triton-X-100, 2 mM DTT, 50% Glycerol)	1.00 $\mu$ l
<i>Tfl</i> DNA Polymerase, 5 units. $\mu$ l <sup>-1</sup> (50% Glycerol, 20 mM Tris HCl pH 8.0, 1 mM DTT, 100 mM KCl, 0.1 mM EDTA, 0.5% Nonidet P40, 0.5% Tween 20)	1.00 $\mu$ l
<b>Total</b>	<b>45.00 <math>\mu</math>l</b>



5. In total, 45  $\mu$ l of master mix cocktails were dispensed into 0.5 ml eppendorf tubes (Eppendorf).
6. Five microlitres of each poliovirus RNA were added into each eppendorf tube containing the pre-formed master mix cocktails in an UV cabinet, to give a final volume of 50  $\mu$ l per reaction.
7. After 30 cycles, 20  $\mu$ l of each PCR product were subjected to agarose (2%) (Seakem) gel electrophoresis (Midicell Primo Gel Apparatus).
8. Poliovirus vaccine strains displayed base pair bands at 440 bp and this region was sequenced using the automated sequencer (Inqaba Biotechnical Industries Pty [Ltd], Pretoria, South Africa).

**B. A RT-PCR for the VP1 capsid-encoding region of poliovirus vaccine strains**

**(Divizia *et al.*, 1999; Guillot *et al.*, 2000):**

1. The RT-PCR was performed as described by Divizia *et al.* (1999) and Guillot *et al.* (2000) with a few modifications.
2. The primers used for the VP1 region were as follows: UG1 (nt 2402 to 2422) and UC1 (nt 2881 to 2862) (Sigma-Genosys).
3. Optimised final concentrations in a total volume of 50  $\mu$ l were: AMV/*Tfl* Reaction Buffer (1x), 2.0 mM MgSO<sub>4</sub>, dNTP Mix (final concentration of 0.2 mM), 10 pmol each of primers UG1 and UC1 (Sigma-Genosys), 5 U each of AMV Reverse Transcriptase and *Tfl* DNA Polymerase (Promega Corp.).
4. The amplification was performed in 30 cycles in a Mini Thermocycler (MJ Research, USA).

**PCR conditions applied:**

42°C for 45 min, reverse transcription

95°C for 30 s, DNA denaturation

50°C for 45 s, primer annealing

72°C for 1 min, primer extension

72°C for 10 min, final extension

} 30 cycles

**Preparation of master mix cocktails:**

Reagents	Master mix per reaction
Nuclease-Free H <sub>2</sub> O	28.80 µl
AMV/ <i>Tfl</i> 1x Reaction Buffer	10.00 µl
dNTP Mix, 10 mM (10 mM each of dATP, dCTP, dGTP, dTTP)	1.00 µl
UG1 (10 pmol)	0.10 µl
UC1 (10 pmol)	0.10 µl
MgSO <sub>4</sub> , 25 mM	3.00 µl
AMV Reverse Transcriptase, 5 units.µl <sup>-1</sup> (0.1 M Potassium phosphate pH 7.2, 0.2% Triton-X-100, 2 mM DTT, 50% Glycerol)	1.00 µl
<i>Tfl</i> DNA Polymerase, 5 units.µl <sup>-1</sup> (50% Glycerol, 20 mM Tris HCl pH 8.0, 1 mM DTT, 100 mM KCl, 0.1 mM EDTA, 0.5% Nonidet P40, 0.5% Tween 20)	1.00 µl
<b>Total</b>	<b>45.00 µl</b>

5. In total, 45 µl of master mix cocktails were dispensed into 0.5 ml eppendorf tubes (Eppendorf).
6. Five microlitres of each poliovirus RNA were added into each eppendorf tube containing the pre-formed master mix cocktails in an UV cabinet, to give a final volume of 50 µl per reaction.
7. After 30 cycles, 20 µl of each PCR product were subjected to agarose (2%) (Seakem) gel electrophoresis (Midicell Primo Gel Apparatus).
8. Poliovirus vaccine strains displayed a 480 bp product.
9. A nested PCR was performed as described by Divizia *et al.* (1999) with a few modifications immediately after completion of the RT-PCR step.
10. The primers used for the nested PCR were as follows: N2426 (nt 2426 to 2446) and N2812 (nt 2812 to 2792) (Sigma-Genosys).
11. The nested PCR mixture contained the following: 1x PCR buffer (10 mM Tris-HCl, pH9; 50 mM KCl; 0.1% Triton X-100), MgCl<sub>2</sub> (final concentration of 1.5 mM), dNTP mix (final concentration of 0.2 mM), primers N2426 and N2812 (10 pmol each) (Sigma-Genosys) and 1.5 U of *Taq* DNA Polymerase (Promega Corp.).
12. Cycling was carried out 30 times in a Mini Thermocycler (MJ Research).

**Nested PCR conditions applied:**

94°C for 2 min, DNA denaturation  
 94°C for 1 min, DNA denaturation  
 45°C for 1 min, primer annealing  
 72°C for 1 min, primer extension  
 72°C for 10 min, final extension

} 30 cycles

**Preparation of master mix cocktails:**

Reagents	Master mix per reaction
Nuclease-Free H <sub>2</sub> O	37.80 µl
1x PCR buffer	5.00 µl
MgCl <sub>2</sub>	4.00 µl
dNTP Mix, 10 mM (10 mM each of dATP, dCTP, dGTP, dTTP)	1.00 µl
N2426 (10 pmol)	0.10 µl
N2812 (10 pmol)	0.10 µl
<i>Taq</i> DNA Polymerase, 5 units.µl <sup>-1</sup> (50% Glycerol, 20 mM Tris HCl pH 8.0, 1 mM DTT, 100 mM KCl, 0.1 mM EDTA, 0.5% Nonidet P40, 0.5% Tween 20)	1.00 µl
<b>Total</b>	<b>49.00 µl</b>

13. In total, 49 µl of master mix cocktails were dispensed into 0.5 ml eppendorf tubes (Eppendorf).
14. One microlitre of each PCR product was added into each eppendorf tube containing the pre-formed master mix cocktails in an UV cabinet, to give a final volume of 50 µl per reaction.
15. After 30 cycles, 20 µl of each nested PCR product was subjected to agarose (2%) (Seakem) gel electrophoresis (Midicell Primo Gel Apparatus).
16. Poliovirus vaccine strains displayed base pair bands at 387 bp and this region was sequenced using the automated sequencer (Inqaba Biotechnical Industries).

**C. Cloning PCR products with pGEM®-T Easy vectors (Promega Corp.). Ligation using 2x rapid ligation buffer**

1. The pGEM®-T Easy Vector and Control Insert DNA (Promega Corp.) were briefly centrifuged in order to collect the contents at the bottom of each tube.
2. The ligation reactions were prepared by mixing vigorously the 2x rapid ligation buffer (LB) (Promega Corp.). A 0.5 ml tube (Eppendorf) known to have low DNA-binding capacity was used in each ligation step.

- The reactions were mixed by pipetting and incubated for 1 h at room temperature ( $\pm 25^{\circ}\text{C}$ ). Alternatively, the reactions can be incubated overnight at  $4^{\circ}\text{C}$  in order to obtain the maximum number of transformants.

Reagents	Standard reaction	Positive control	Negative control
2x rapid ligation buffer	5 $\mu\text{l}$	5 $\mu\text{l}$	5 $\mu\text{l}$
pGEM®-T Easy vector (50 ng)	1 $\mu\text{l}$	1 $\mu\text{l}$	1 $\mu\text{l}$
PCR product	X $\mu\text{l}$	-	-
Control insert DNA	-	2 $\mu\text{l}$	-
T4 DNA ligase (3 units per L)	1 $\mu\text{l}$	1 $\mu\text{l}$	1 $\mu\text{l}$
Deionised water to a final volume of:	10 $\mu\text{l}$	10 $\mu\text{l}$	10 $\mu\text{l}$

### Preparation of LB medium

- The following reagents were mixed with 1 L of distilled water in a 1 L Erlenmeyer flask:  $10\text{ g}\cdot\text{L}^{-1}$  Bacto tryptone (Difco Laboratories, Detroit, USA),  $10\text{ g}\cdot\text{L}^{-1}$  Bacto yeast extract (Difco) and  $5\text{ g}\cdot\text{L}^{-1}$  NaCl (Difco).
- The pH was adjusted to pH 7.

### Preparation of LB/ampicillin/ITPG/X-Gal plates

- LB medium was prepared by mixing LB broth (Difco) and 15 g of agar (Difco), the top of the flask was wrapped with aluminium foil and autoclaved.
- The LB medium was incubated at  $50^{\circ}\text{C}$  in order to prevent solidification of the medium.
- The medium was heated to  $50^{\circ}\text{C}$  at which the following substances were added: 400  $\mu\text{l}$  ampicillin (Mast Diagnostics, Mast group Ltd, Merseyside, United Kingdom), 400  $\mu\text{l}$  ITPG and 400  $\mu\text{l}$  X-Gal (Promega Corp.). The medium was gently mixed by swirling to avoid formation of air bubbles.
- Approximately, 20 ml of the medium was poured in each Petri dish near a burner in a sterile area. The plates were allowed to cool to room temperature ( $25^{\circ}\text{C}$ ) before use.
- The plates were stored at  $4^{\circ}\text{C}$  till further analysis.

### Modified transformation of JM109 (Promega Corp.) high efficiency competent cells

- LB/ampicillin/ITPG/X-Gal plates were prepared.
- The ligation reaction was briefly vortexed.

3. Each ligation reaction (10  $\mu$ l) was pipetted into a sterile 1.5 ml tube (Eppendorf) on ice. The JM109 (Promega Corp.) high efficiency competent cells were incubated on ice until thawed (5 min).
4. The cells were gently mixed by flicking the tube. Cells (100  $\mu$ l) were carefully pipetted to the ligation reaction tubes.
5. The tubes were gently mixed and incubated on ice for 20 min.
6. The cells were heat-shocked for 45-50 s in a heat block (QBT2, Grant Instruments, Cambridge, United Kingdom) at exactly 42°C and tubes were not shaken.
7. The tubes were immediately put back on ice for 2 min. LB broth (900  $\mu$ l) was added to the ligation reaction transformations at room temperature ( $\pm$ 25°C).
8. The tubes were incubated for 1 h at 37°C in a shaking incubator (Labcon, Labotec, South Africa) at 200 rpm.
9. Each transformation culture (100  $\mu$ l) was plated onto duplicate LB/ampicillin/IPTG/X-Gal plates. The plates were incubated overnight at 37°C.
10. The white colonies were selected and each colony represented one transformation.

#### **Screening of transformed (white) colonies with PCR for inclusion of DNA fragments**

1. The white colonies were diluted in 20  $\mu$ l of nuclease-free water (Promega Corp.).
2. A 45  $\mu$ l reaction volume for the PCR containing the following was prepared: AMV/*Tfl* Reaction Buffer (1x), 2.0 mM MgSO<sub>4</sub>, dNTP Mix (final concentration of 0.2 mM), 10 pmol each of primers UG52 and UC53, 5 U each of AMV Reverse Transcriptase and *Tfl* DNA Polymerase (Promega Corp.), and finally 5  $\mu$ l of transformed (white) colonies suspension.
3. The amplification was performed in 30 cycles in a Mini Thermocycler (MJ Research). The PCR conditions were as follows: reverse transcription for 45 min at 42°C, denaturation for 30 s at 95°C, annealing for 45 s at 45°C, extension for 1 min at 72°C and after 30 cycles final extension for 10 min at 72°C.

#### **Preparation for use of QIAprep spin miniprep kit protocol (Qiagen)**

##### *a) Growing culture*

1. LB broth (10 ml) was mixed with 10  $\mu$ l ampicillin and with 5  $\mu$ l of the colony suspensions (found positive when screening with UG52 and UC53 primers).

2. The suspension was incubated overnight (18-24 h) at 37°C with agitation. Cells were pelleted by centrifugation at 1 000 x g for 10 min (Eppendorf Centrifuge 5402D) at ±25°C.

*b) Preparation of reagents*

1. The RNase A solution was added to buffer P1, mixed and stored at 2-8°C.
2. Absolute ethanol (Merck) was added to buffer PE.
3. Buffers P2 and N3 were investigated for salt precipitation before use. The precipitate was dissolved by warming solution to 37°C.
4. Buffer P2 should not be shaken vigorously. Buffer P2 was closed immediately after use to avoid acidification from CO<sub>2</sub> in the air.

*c) QIAprep spin miniprep kit protocol*

1. The pelleted bacterial cells were re-suspended in 250 µl of buffer P1 and transferred to a microcentrifuge tube (Eppendorf).
2. Buffer P2 (250 µl) was added and the tube was gently inverted 4-6 times to mix the suspension. The suspension was not vortexed as this would result in shearing of genomic DNA.
3. Buffer N3 (350 µl) was added and the tube was inverted gently 4-6 times. To avoid localized precipitation, the solution was mixed gently but thoroughly, immediately after addition of buffer N3. The solution should become cloudy.
4. The suspension was centrifuged for 10 min at 10 000 x g (Eppendorf Centrifuge 5402D) at ±25°C.
5. The supernatants were pipetted into the QIAprep column. The suspension was centrifuged for 60 s at 10 000 x g (Eppendorf Centrifuge 5402D) at ±25°C. The flow-through was discarded.
6. The QIAprep spin column was washed by adding 0.5 ml buffer PB and centrifuged for 60 s at 10 000 x g (Eppendorf Centrifuge 5402D) at ±25°C. The flow-through was discarded.
7. The QIAprep spin column was washed by adding 0.75 ml buffer PE and centrifuged for 60 s at 10 000 x g (Eppendorf Centrifuge 5402D) at ±25°C.
8. The flow-through was discarded and centrifuged for an additional 1 min at 10 000 x g (Eppendorf Centrifuge 5402D) at ±25°C to remove residual wash buffer.

9. The QIAprep spin column was placed in a clean 1.5 ml microcentrifuge tube (Eppendorf). To elute, 50 µl diluted buffer EB (1 µl EB: 7 µl water; 80°C) was added to the center of each QIAprep spin column, the tubes were incubated for 1 min at room temperature ( $\pm 25^{\circ}\text{C}$ ) and centrifuged for 1 min at 10 000 x g (Eppendorf Centrifuge 5402D).
10. The elute was used for sequencing of DNA samples.

**D. Sequencing using the automated sequencer (Inqaba Biotechnical Industries)**

1. The exonuclease I/Shrimp alkaline phosphatase (Fermentas, Vilnius, Lithuania) was used to clean the PCR samples from primers and nucleotides.
2. The ABI BigDye Terminator cycle sequencing kit version 3.1 was used to sequence the PCR products.
3. In the latter procedure, approximately 500 ng of PCR product and 0.8 pmol of each of the same primers used in the amplification reaction were employed in each sequencing reaction. Both strands of the amplified fragments were sequenced to confirm the nature of the product obtained.
4. The data was analysed on a Spectrumedix SCE2410 genetic analysis system.

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