

Aug 06, 2025

Tiled PCR Amplification for Poliovirus Whole-Genome Sequencing

DOI

<https://dx.doi.org/10.17504/protocols.io.5qpvwjr7l4o/v1>

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Protocol Citation: Kafayat Arowolo, Dimitra Klapsa, Manasi Majumdar, Erika Bujaki, Alison Tedcastle, Javier Martin 2025. Tiled PCR Amplification for Poliovirus Whole-Genome Sequencing. **protocols.io**

<https://dx.doi.org/10.17504/protocols.io.5qpvwjr7l4o/v1>

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Protocol status: Working

We use this protocol and it's working



Created: June 23, 2025

Last Modified: August 06, 2025

Protocol Integer ID: 220740

Keywords: genome sequencing of poliovirus, pcr amplification for poliovirus whole, poliovirus whole, full poliovirus, poliovirus, robust recovery of full poliovirus, viral rna via rt, viral rna, genome sequencing this protocol, genome sequencing, amplicon strategy, tiled pcr amplification, tiled amplicon strategy, amplicon, environmental rna extract, overlapping pcr product, pcr, pcr product, amplification

Funders Acknowledgements:

BMGF

Grant ID: INV-043859

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Abstract

This protocol describes the amplification and whole-genome sequencing of poliovirus from clinical or environmental RNA extracts using an overlapping tiled amplicon strategy. Four overlapping PCR products are generated from viral RNA via RT-PCR. Amplicons are pooled and purified before library preparation for Oxford Nanopore or Illumina sequencing. This protocol enables robust recovery of full poliovirus genomes for surveillance and research purposes.

Guidelines

Clean reagents should be stored away from any biological samples and viral materials and handled in a dedicated biological safety cabinet or work area. Personal protective equipment such as lab coats, gloves and goggles, laboratory equipment including pipettes and pipettors/pipetting aids, tube racks and packs of consumables should only be used in their designated work area.

Label all reagent boxes with date of receipt and individual containers with date of opening. To avoid confusion, keep reagents intended to use for this protocol labelled and well separated from other reagents even if they are from the same manufacturer.

Keep working reagents and reagent aliquots in clearly labelled containers during the whole protocol. Ensure that pipettes, racks and work surfaces are cleaned using an appropriate surface decontaminant or cleaning agent (i.e. RNase away, RNaseZap) is used to avoid contamination and to protect viral RNA.

In Post-PCR areas specific cleaning agent (i.e. DNAway) should be used to decontaminate tools and surfaces prior to starting as well as after finishing using a work area. Use filtered pipette tips only. Microtubes, PCR strips and pipette tips should be certified Nuclease free/PCR grade.

Reagent preparations

1 Viral RNA extraction

We recommend using the MagMAX Viral RNA Extraction. Please follow the appropriate protocol depending on your starting material and manual or automated workflow:

Purification of viral RNA from cell culture isolates or FTA cards using MagMAX Viral RNA Isolation Kit - <https://dx.doi.org/10.17504/protocols.io.x54v928mml3e/v2>

Small volume viral RNA extraction using MagMAX Viral RNA Isolation Kit - <https://dx.doi.org/10.17504/protocols.io.81wgbzpyogpk/v2>

- 1.1 Using freshly extracted viral RNA is recommended whenever possible to facilitate successful amplification of long genome fragments.

Repeated freeze-thaw of samples or purified RNA can affect viral nucleic acid integrity, reducing amplification efficiency.

2 Primer stocks

The following primers are used to amplify 4 overlapping tiles along the poliovirus genome:

Tile	Name	Sequence (5'-3')	Product size
1	PanPV_WG_Fwd	CCAGAGGCCACGTGGCGGCTAG	~2 kb
	PV_1999_Rev	CAAGTTGAGGGTATCATGGTGT	
2	PanSabin_Seg2_Fwd	TCTGCCCCRGTKGATTAYCTC	~ 2 kb
	Q8_Rev	AAGAGGTCTCTRTCCACAT	
3	PanSabin_Seg3_Fwd	GTMAATGATCACAACCC	~2.5 kb
	PanSabin_Seg3_Rev	GTTGGAAAGTTGTACATTAG	
4	PV_5455_For	GGRCAYCARGGWGCATAYACTG	~2 kb
	PanPV_WG_Rev	CCGAATTAAGAAAAATTTACCCCTACA	

Tiled poliovirus PCR primers

IUPAC codes: R = A/G, Y = C/T, K = G/T, W = A/T, M = A/C

- 2.1 Prepare individual working primer stocks at 10 µM. Store stocks at -20 °C.

3 Experiment controls

We recommend using assay positive and negative controls with additional controls introduced in subsequent steps to indicate potential problems and help with troubleshooting when necessary.

RNA extraction should ideally include a known positive and a negative control. A no-template PCR control should be added in the RT-PCR step. Inclusion of additional RT-PCR positive control and a library preparation/sequencing control should also be considered, especially during problem solving.

Tiles RT-PCR reactions

4 Preparation of RT Master Mixes.

Note

We recommend using the SuperScript III One-Step RT-PCR system with Platinum Taq High Fidelity DNA Polymerase (Product number: 12574035). When choosing an alternative equivalent product consider enzyme sensitivity, fidelity and efficiency with long amplicons. Adjust reaction mix component volumes and cycling conditions accordingly.

- 4.1 Prepare a master mix for each Tiled PCR reaction in labelled micro-centrifuge tubes using the reagent volumes detailed in the table below for the number of samples you have including controls plus 1-2 reactions extra to cover pipetting loss. The reaction mix and enzyme mix are provided in the Super Script RT-PCR kit.

	A	B
	Reagent	1 reaction (µL)
	2x Reaction Mix (10x)	12.5
	Reverse Primer	1
	SSIII Platinum Taq HiFi enzyme mix	1
	Nuclease Free Water	3.5

A	B
total volume:	18

RT reaction Master Mix

- 4.2 Vortex each Master Mix for 3 seconds and spin down for 5 seconds to gather contents at the bottom of the tube.
- 4.3 Aliquot 18µL Master Mix to each labelled 0.2mL PCR tube/strip/plate.
- 4.4 Add 6µL of viral RNA or control to each PCR tube and close carefully.
- 4.5 Mix reactions gently by flicking and centrifuge briefly to collect content.
- 4.6 Incubate reactions in thermocycler for RT step as follows:
50°C for 30 minutes, 10 °C hold.
- 4.7 Add 1µL of Forward Primer (10µM) to each reaction using individual tips and close tubes carefully.
- 4.8 Amplify using the following cycling conditions:

A	B	C	D
Cycle	Step	Temperature (°C)	Time
1	Initial denaturing	94	2 minutes
42	Denaturing	94	15 seconds
	Annealing	50	30 seconds
	Extension	68	3 minutes
1	Final extension	68	5 minutes
-	Hold	10	-

PCR cycling conditions



Product verification

- 5 Perform electrophoresis using preferred method (i.e. agarose gel electrophoresis, Tapestation, Bioanalyzer) to confirm PCR products. Approximate expected amplicon sizes are shown in Step 2. in this protocol.

Amplicon pooling, purification and quantification

6

Note

We present a simplified approach here directed by electrophoresis results with purifying and quantifying the pooled reactions. Alternatively, each amplicon can be purified and quantified individually for accurate equimolar pooling.

Pool all four amplicons (or as many as were successfully amplified) in approximately equimolar quantities based on electrophoresis results for each virus sample.

- 7 Purify pooled PCR products using either centrifuge columns or magnetic beads following manufacturer's recommendations.

Amplicon pooling, purification and quantification

- 8 Quantify purified DNA using a validated method, ie. Qubit dsDNA Broad Range Kit

Library preparation and sequencing

- 9 Normalise input DNA based on recommendations for the applied sequencing method.

- 9.1 Follow sequencing library preparation steps as per manufacturer's instructions.

For nanopore sequencing with native barcoding method, steps in <https://dx.doi.org/10.17504/protocols.io.e6nvwjko9lmk/v1> can be followed.

Alternative pairings and additional primers

- 10 If the initial 4-tile PCR approach, as described above, fails to yield a full-length viral genome sequence, explore alternative primer pairings to complete the missing

fragments. Consider using serotype or strain-specific primers as necessary. Additional options are listed in the table below.

Reaction	Name	Sequence (5'-3')	Product size
1	nOPV2_1864_Fwd	AACACTCCAGGGAGTAACCAGTA	~1.7 kb
	Q8_Rev	AAGAGGTCTCRTTCACAT	
2	Y7R_Fwd	CGCACRCTGAARTCATRCA	~2 kb
	nOPV_cre_Rev	TCGATACGGTGCTTGGATTAAATTG	
	Ari_Rev	TCAATACGGTGTGCTCTTGAAGT	
3	PanSabin_Seg3_Fwd	GTMAATGATCACACACC	~2.4 kb
	PV_5643_Rev	CWCCWGGTGARGCRTGRGTTGG	
4	PV_4440_Fwd	CAGTCCAAGAGGTTTGCACC	~3 kb
	PanPV_WG_Rev	CCGAATTAAAGAATAAAAATTACCCTCACA	
5	nOPV2_5439_Fwd	CTGGGCACCAGGGTGCATA	~2 kb
	PanPV_WG_Rev	CCGAATTAAAGAATAAAAATTACCCTCACA	
6	Y7R_Fwd	CGCACRCTGAARTCATRCA	~5 kb
	PanPV_WG_Rev	CCGAATTAAAGAATAAAAATTACCCTCACA	
7	PanPV_WG_Fwd	CCAGAGGCCACGTGGCGGCTAG	~3.5 kb
	Q8_Rev	AAGAGGTCTCRTTCACAT	
8	PanSabin_Seg3_Fwd	GTMAATGATCACACACC	~4 kb
	PanPV_WG_Rev	CCGAATTAAAGAATAAAAATTACCCTCACA	

Alternative primer pairings

Note

Remember to adjust PCR extension time in cycling conditions according to amplicon size.