

Jan 12, 2026

Terminal PCR for Amplifying the 5' and 3' Termini of the Monkeypox Virus Genome

DOI

<https://dx.doi.org/10.17504/protocols.io.36wgqdmxvk5/v1>

Masayasu Misu¹, Takeshi Kurosu¹, Tomoki Yoshikawa¹, Madoka Kawahara¹, Kohei Oishi¹, Masayuki Shimojima¹, Hideki Ebihara¹

¹Department of Virology I, National Institute of Infectious Diseases



Tomoki Yoshikawa

National Institute of Infectious Diseases

Create & collaborate more with a free account

Edit and publish protocols, collaborate in communities, share insights through comments, and track progress with run records.

Create free account

OPEN  ACCESS



DOI: <https://dx.doi.org/10.17504/protocols.io.36wgqdmxvk5/v1>

Protocol Citation: Masayasu Misu, Takeshi Kurosu, Tomoki Yoshikawa, Madoka Kawahara, Kohei Oishi, Masayuki Shimojima, Hideki Ebihara 2026. Terminal PCR for Amplifying the 5' and 3' Termini of the Monkeypox Virus Genome . **protocols.io**

<https://dx.doi.org/10.17504/protocols.io.36wgqdmxvk5/v1>

License: This is an open access protocol distributed under the terms of the **Creative Commons Attribution License**, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Protocol status: Working

We use this protocol and it's working

Created: February 04, 2025

Last Modified: January 12, 2026

Protocol Integer ID: 119518

Keywords: termini of the monkeypox virus genome, monkeypox virus genome, terminal sequences of the monkeypox virus, monkeypox virus, viral dna, using standard nucleic acid purification method, standard nucleic acid purification method, read depth at the genome termini, genome termini, amplified dna, purified nucleic acid, generation sequencing, illumina dna prep, comprehensive genome coverage, viral suspension, ensuring comprehensive genome coverage, idt for illumina dna, nucleic acid, illumina dna, terminal pcr, using pcr, sequencing, micrococcal nuclease treatment, genome, rna, virus, oxford nanopore technology, pcr

Funders Acknowledgements:

Ministry of Health, Labor and Welfare of Japan

Grant ID: 20HA2005 and 23HA2004

Japan Agency for Medical Research and Development

Grant ID: 21fk0108426j0001, 25fk0108660h0003, 22fk0108502j0101 and 23fk0108581j0701

Japan Society for the Promotion of Science

Grant ID: JP22K085

Abstract

This protocol describes the amplification and purification of the 5' and 3' terminal sequences of the Monkeypox virus (MPXV) genome using PCR. The method is designed to compensate for the reduced read depth at the genome termini observed with the Nuclease-MDA method, ensuring comprehensive genome coverage.

Purified nucleic acids can be obtained either from viral DNA enriched via the Nuclease-MDA method or directly from viral suspensions extracted using standard nucleic acid purification methods, with or without micrococcal nuclease treatment.

This method was initially optimized for Oxford Nanopore Technologies (ONT) MinION sequencing. However, after completing Section 3, the amplified DNA can be used for Illumina Next-Generation Sequencing (NGS), following standard library preparation protocols.

This protocol can be applied using the ONT Native Barcoding Kit(SQK-NBD114.24, SQK-NBD114.96), Rapid Barcoding Kit V14 (SQK-RBK114.24 or SQK-RBK114.96) or Illumina DNA Prep, (M) Tagmentation (24 Samples, IPB) (Cat#20060060), IDT for Illumina DNA/RNA UD Indexes Set A (Cat#20027213), and the iSeq100 system.

Protocol materials

 High Pure Viral Nucleic Acid Kit **Roche Catalog #11858874001**

 DNA LoBind Tube 1.5ml **Eppendorf Catalog #022431021**

 Agencourt AMPure XP **Beckman Coulter Catalog #A63880**

 NEBNext Quick Ligation Module - 20 rxns **New England Biolabs Catalog #E6056S**

 Blunt/TA Ligase Master Mix - 50 rxns **New England Biolabs Catalog #M0367S**

 Qubit dsDNA BR (Broad Range) assay **Thermo Fisher Scientific Catalog #Q32850**

 NEBNext Ultra II End Repair/dA-Tailing Module - 24 rxns **New England Biolabs Catalog #E7546S**

 KOD One® PCR Master Mix **Toyobo Catalog #KMM-101**



The viral genomic DNA extraction

1 The viral genomic DNA extraction is performed using

 High Pure Viral Nucleic Acid Kit **Roche Catalog #11858874001** .

1.1 Add  200 μL of binding buffer,  4 μL PolyA carrier RNA and  50 μL Proteinase K.

Mix well by pipetting and inverting the tube thoroughly, and spin down.

 72 °C  00:10:00

1.2 Add  100 μL of binding buffer and mix well by pipetting or inverting the tube thoroughly, and spin down.

1.3 Transfer the whole sample to a High Pure Filter Tube.

 8000 x g, Room temperature, 00:01:00

Discard the flow-through liquid and Collection Tube, and insert the Filter Tube into a new Collection Tube.

1.4 Add  500 μL of inhibitor removal buffer.

 8000 x g, Room temperature, 00:01:00

Discard the flow-through liquid and Collection Tube, and insert the Filter Tube into a new Collection Tube.

1.5 Add  450 μL of wash buffer.

 8000 x g, Room temperature, 00:01:00

Discard the flow-through liquid and Collection Tube, and insert the Filter Tube into a new Collection Tube.

1.6 Add  450 μL of wash buffer.

 13000 x g, Room temperature, 00:01:00 and discard the flow-through liquid.

Discard the Collection Tube and insert the Filter Tube into a 1.5 ml tube -

 DNA LoBind Tube 1.5ml **Eppendorf Catalog #022431021** .

1.7 Add  50 μL elution Buffer.

 13000 x g, Room temperature, 00:01:00

Note

The purified  50 µL viral genomic DNA can be stored at -80°C.

PCR reaction

- 2 The purified viral genomic DNA is amplified by PCR using the following primer set.

	Target	5' Primer Name	5' Primer sequence	3' Primer name	3' Primer sequence	Approx. product size (kb)
	5' terminal	MPXV term	GTGTGACCCACGA CCGTAG	MPXV_5-inner	TCCATCTCCCTC TGGACCAC	8
	3' terminal	MPXV_3-inner	AATCGTTCTCCTC GGTGTA	MPXV term	GTGTGACCCACG ACCGTAG	9

- 2.1 PCR reaction components are as follows:

For 5' terminal: total  30 µL reaction

-  15 µL  KOD One® PCR Master Mix **Toyobo Catalog #KMM-101**
-  1.5 µL  5 micromolar (µM) MPXV term
-  1.5 µL  5 micromolar (µM) MPXV_5-inner
-  11 µL H₂O
-  1 µL purified viral genomic DNA

For 3' terminal: total  30 µL reaction

-  15 µL  KOD One® PCR Master Mix **Toyobo Catalog #KMM-101**
-  1.5 µL  5 micromolar (µM) MPXV term
-  1.5 µL  5 micromolar (µM) MPXV_3-inner
-  11 µL H₂O
-  1 µL purified viral genomic DNA

PCR Conditions are as follows (same as 5' and 3' terminals):



	Step	Temperature (°C)	Time (sec)	Cycles
	Initial Denaturation	98	15	
	Denaturation	98	10	5
	Annealing	65 (-1°C per cycle)	5	
	Extension	68	90	
	Denaturation	98	10	35
	Annealing	60	5	
	Extension	68	90	

Note

Expected PCR product sizes are ~8 kb for the 5' end and ~9 kb for the 3' end, which can be confirmed using gel electrophoresis or a Bioanalyzer.

DNA purification by AMPure XP

3 The DNA is purified using

 Agencourt AMPure XP **Beckman Coulter Catalog #A63880**

3.1 Add  15 µL (X0.5 volume) AMPure beads

3.2 Incubate  00:05:00  Room temperature

3.3 Spin down and pellet on a magnet.

Wait for  00:01:00 and pipette off the supernatant.

3.4 Wash twice by  100 µL 70 % ethanol, remove the ethanol using a pipette, and discard.

3.5 Spin down and pipette off any residual ethanol.



- 3.6 Resuspend pellet in  40 μL nuclease-free H_2O .
Incubate  00:05:00  37 $^{\circ}\text{C}$ and tapping occasionally.
- 3.7 Spin down and pellet the beads on the magnet until the elute is clear and colorless.

- 3.8 Remove and retain  40 μL elute into a new tube.

- 4 DNA concentration is measured using a Qubit 4 Fluorometer with

 Qubit dsDNA BR (Broad Range) assay **Thermo Fisher Scientific Catalog #Q32850**

-  199 μL 1X working solution
-  1 μL DNA

Mix by vortexing.

Incubate  00:02:00  Room temperature and measure.

Note

At this step, whole-genome sequencing using the ONT Rapid Barcoding Kit V14 (SQK-RBK114.24 or SQK-RBK114.96) or Illumina's next-generation sequencer is also possible. In this case, the subsequent steps should follow the ONT Rapid Barcoding Kit or Illumina's library preparation protocol. We have confirmed that this protocol works using the Illumina DNA Prep, (M) Tagmentation (24 Samples, IPB) (Cat#20060060) and IDT for Illumina DNA/RNA UD Indexes Set A, Tagmentation (Cat#20027213).

DNA end-prep

10m

- 5 The purified DNA is end-prepped using

 NEBNext Ultra II End Repair/dA-Tailing Module - 24 rxns **New England Biolabs Catalog #E7546S**

Note

The molar concentration of the DNA sample can be converted based on the amplicon length (9 kb).



5.1 Total 15 µl reaction

10m

- 12.5 µL amplicon DNA (1100 ng for 9 kb amplicons)
- 1.75 µL Ultra II end-prep reaction buffer
- 0.75 µL Ultra II end-prep reaction Mix

Mix by pipetting and spin down.

20 °C 00:05:00

65 °C 00:05:00

6 The DNA is purified using

Agencourt AMPure XP Beckman Coulter Catalog #A63880 .

Add 7.5 µL (X0.5 volume) AMPure beads

[go to step #3](#)

Resuspend pellet in 6 µL nuclease-free H₂O.

Note

The DNA can be stored at 4°C overnight.

Native barcode ligation

- 7 The end-prepped DNA is ligated with native barcode using **Native Barcoding Kit V14 - Oxford Nanopore Technologies Catalog #SQK-NBD114.24 or #SQK-NBD114.24** with **Blunt/TA Ligase Master Mix - 50 rxns New England Biolabs Catalog #M0367S** .

7.1 Total 13 µl reaction

- 5 µL End-prepped DNA
- 1.5 µL native barcode
- 6.5 µL Blunt/TA ligase master mix

Mix by pipetting and spin down.

Room temperature 00:20:00

7.2

Add the following volume of EDTA to each well and mix thoroughly by pipetting and spin down briefly.

**Note**

Ensure you follow the instructions for the cap colour of your EDTA tube.

	EDTA cap colour	Volume per well
	For clear cap EDTA	1.3 μ l
	For blue cap EDTA	2.6 μ l

EDTA is added at this step to stop the reaction.

8 Pool all the barcoded samples in a 1.5 ml Eppendorf DNA LoBind tube.

9 The DNA is purified using

 Agencourt AMPure XP **Beckman Coulter Catalog #A63880**

Add  5.7 or 6.5 μ L (X0.4 volume) AMPure beads multiply by the original number of tubes.

9.1 Incubate on rotor mixer.

 00:05:00  Room temperature

9.2 Spin down and pellet on a magnet.

Wait for  00:01:00 and pipette off the supernatant.

9.3 Wash twice by  700 μ L 70 % ethanol, remove the ethanol using a pipette, and discard.

9.4 Spin down and pipette off any residual ethanol.

9.5 Resuspend the pellet in 15 μ L of nuclease-free H₂O when the number of pooled samples is fewer than 8.

If 8 or more samples are pooled, resuspend the pellet in 35 μ L of nuclease-free H₂O.

Incubate on a rotor mixer.

 00:10:00  37 °C

Every 2 minutes, agitate the sample by gently flicking for 10 seconds to encourage DNA elution.

9.6 Spin down and pellet the beads on the magnet until the elute is clear and colorless.



9.7 Remove and retain the elute into a new tube.

Adapter ligation and clean-up

10 Adaptor Ligation with pooled samples is performed using **Ligation Sequencing Kit - Oxford Nanopore Technologies Catalog #SQK-NBD114.24 or #SQK-NBD114.96 with**

NEBNext Quick Ligation Module - 20 rxns **New England Biolabs Catalog #E6056S**

10.1 Total 20 µl reaction

- 12 µL DNA
- 2 µL Native Adapter (NA)
- 4 µL NEB Next Quick Ligation Reaction Buffer(5X)
- 2 µL Quick T4 DNA ligase

Mix gently and incubate.

Room temperature 00:20:00

11 The adaptor-ligated DNA is purified using

Agencourt AMPure XP **Beckman Coulter Catalog #A63880** .

Prepare AMPure XP beads for use; resuspend by vortexing.

Transfer amplified DNA sample to 1.5ml low binding tube.

11.1 Add 10 µL (X0.5 volume) AMPure XP reagent and mix by pipetting.

Incubate 00:05:00 Room temperature

11.2 Spin down and pellet on a magnet. Wait for 00:01:00 and pipette off the supernatant.

11.3 ▪ Wash twice by 100 µL Long Fragment Buffer (LFB) and remove the LFB using a pipette and discard.

11.4 Spin down and pipette off any residual LFB.

11.5 ▪ Resuspend pellet in 15 µL Elution Buffer (EB)

00:05:00 37 °C and tapping occasionally.

11.6 Spin down and pellet the beads on the magnet until the elute is clear and colourless.



11.7 Remove retain  15 μL elute into a new tube.

12 DNA concentration is measured using a Qubit 4 Fluorometer with

 Qubit dsDNA BR (Broad Range) assay **Thermo Fisher Scientific Catalog #Q32850**

-  199 μL 1X working solution
-  1 μL DNA

Mix by vortexing.

Incubate  00:02:00  Room temperature and measure.

Note

The molar concentration of the DNA sample can be converted based on the amplicon length (9 kb).

13 Make up the library to  12 μL at 10-20 fmol

Sequencing by MinION

14 Sequencing according to the manufacturer's instructions.