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

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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 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
- Qubit dsDNA BR (Broad Range) assay **Thermo Fisher Scientific** **Catalog #**Q32850
- Agencourt AMPure XP **Beckman Coulter** **Catalog #**A63880
- Agencourt AMPure XP **Beckman Coulter** **Catalog #**A63880
- NEBNext Quick Ligation Module - 20 rxns **New England Biolabs** **Catalog #**E6056S
- Agencourt AMPure XP **Beckman Coulter** **Catalog #**A63880
- 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
- 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℃.

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 seuence	Approx. product size (kb)
	5′ terminal	MPXV term	GTGTGACCCACG ACCGTAG	MPXV_5-inner	TCCATCTCCCTC TGGACCAC	8
	3′ terminal	MPXV_3-inner	AATCGTTCTCCTC GGTGTCA	MPXV term	GTGTGACCCAC GACCGTAG	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 [M] 5 micromolar (µM) MPXV term
- 1.5 µL [M] 5 micromolar (µM) MPXV_5-inner
- 11 µL H_2O
- 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 [M] 5 micromolar (µM) MPXV term
- 1.5 µL [M] 5 micromolar (µM) MPXV_3-inner
- 11 µL H_2O
- 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 °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
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- 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 Illumina's next-generation sequencer is also possible. In this case, the subsequent steps should follow 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 amplicoms)
- 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 H_2O.

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 µl
	For blue cap EDTA	2.6 µ

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 µL (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 µL 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 µL of nuclease-free H_2O when the number of pooled samples is fewer than 8.
If 8 or more samples are pooled, resuspend the pellet in 35 µL of nuclease-free H_2O.
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.