Nuclease-MDA for Enriching Monkeypox Virus Genomes for NGS

Masayasu Misu[1], Takeshi Kurosu[1], Tomoki Yoshikawa[1], Madoka Kawahara[1], Kohei Oishi[1], Masayuki Shimojima[1], Hideki Ebihara[1]

[1]Department of Virology I, National Institute of Infectious Diseases

Tomoki Yoshikawa
National Institute of Infectious Diseases

Protocol Info: Masayasu Misu, Takeshi Kurosu, Tomoki Yoshikawa, Madoka Kawahara, Kohei Oishi, Masayuki Shimojima, Hideki Ebihara . Nuclease-MDA for Enriching Monkeypox Virus Genomes for NGS. **protocols.io https://protocols.io/view/nuclease-mda-for-enriching-monkeypox-virus-genomes-cvxtw7nn**

Created: June 20, 2023

Last Modified: August 29, 2025

Protocol Integer ID: 83667

Keywords: Oxford Nanopore Technology, RNA virus, Sequence method, MinION, Nanopore sequencing, RCA-NGS

Abstract

This protocol describes the enrichment of the monkeypox virus (MPXV) genome using the Nuclease-MDA method, optimized for Oxford Nanopore Technologies (ONT) sequencing with V14 chemistry.

The method can also be adapted for Illumina sequencing following Section 7 of this protocol. The protocol has been validated using Illumina DNA Prep, (M) Tagmentation (Cat#20060060), IDT for Illumina DNA/RNA UD Indexes Set A (Cat#20027213), and the iSeq100 platform.

This approach eliminates unwanted host DNA via micrococcal nuclease treatment, preserving the intact viral genome. The viral genome is then amplified using multiple displacement amplification (MDA), an isothermal whole-genome amplification (WGA) technique employing Phi29 polymerase. This method provides a high proportion of viral reads (~90%) without requiring specific PCR or virus concentration steps.

This method enables high-depth whole-genome sequencing of MPXV and can be readily adapted to other poxviruses, such as vaccinia virus and cowpox virus, with appropriate primer modifications.

Materials

- Micrococcal Nuclease - 320,000 gel units **New England Biolabs Catalog #**M0247S
- High Pure Viral Nucleic Acid Kit **Roche Catalog #**11858874001
- **Dr.GenTLE Precipitation Carrier - Takara Catalog #9094**
- Agencourt AMPure XP **Beckman Coulter Catalog #**A63880
- **GenomiPhi V3 Ready-To-Go DNA Amplification Kit - Cytiva Catalog #25-6601-24**
- T7 Endonuclease I - 250 units **New England Biolabs Catalog #**M0302S
- NEBNext FFPE DNA Repair Mix - 24 rxns **New England Biolabs Catalog #**M6630S
- NEBNext Ultra II End Repair/dA-Tailing Module - 24 rxns **New England Biolabs Catalog #**E7546S
- Blunt/TA Ligase Master Mix - 50 rxns **New England Biolabs Catalog #**M0367S
- NEBNext Quick Ligation Module - 20 rxns **New England Biolabs Catalog #**E6056S
- **Native Barcoding Kit 24 V14 - Oxford Nanopore Technologies Catalog #SQK-NBD114.24**
- Qubit 4 Fluorometer **Thermo Fisher Scientific Catalog #**Q33238
- Qubit 1X dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #**Q33230
- DNA LoBind Tube 1.5ml **Eppendorf Catalog #**022431021
- 0.2 ml PCR Tube strips **Eppendorf Catalog #**0030124359
- **100 % ethanol**
- **70 % ethanol**
- **TE(pH8.0)**
- **nuclease-free H_2O**

Protocol materials

- Micrococcal Nuclease - 320,000 gel units **New England Biolabs** **Catalog #**M0247S
- T4 RNA Ligase 2, truncated KQ - 2,000 units **New England Biolabs** **Catalog #**M0373S
- Superscript IV **Thermo Fisher Scientific** **Catalog #**18090050
- 0.2 ml PCR Tube strips **Eppendorf** **Catalog #**0030124359
- NEBNext FFPE DNA Repair Mix - 24 rxns **New England Biolabs** **Catalog #**M6630S
- NEBNext Ultra II End Repair/dA-Tailing Module - 24 rxns **New England Biolabs** **Catalog #**E7546S
- Agencourt AMPure XP **Beckman Coulter** **Catalog #**A63880
- High Pure Viral Nucleic Acid Kit **Roche** **Catalog #**11858874001
- NEBNext Ultra II End Repair/dA-Tailing Module - 24 rxns **New England Biolabs** **Catalog #**E7546S
- Qubit 4 Fluorometer **Thermo Fisher Scientific** **Catalog #**Q33238
- Qubit 1X dsDNA HS Assay Kit **Thermo Fisher Scientific** **Catalog #**Q33230
- Micrococcal Nuclease - 320,000 gel units **New England Biolabs** **Catalog #**M0247S
- DNA LoBind Tube 1.5ml **Eppendorf** **Catalog #**022431021
- 0.2 ml PCR Tube strips **Eppendorf** **Catalog #**0030124359
- Agencourt AMPure XP **Beckman Coulter** **Catalog #**A63880
- T7 Endonuclease I - 250 units **New England Biolabs** **Catalog #**M0302S
- NEBNext FFPE DNA Repair Mix - 24 rxns **New England Biolabs** **Catalog #**M6630S
- Blunt/TA Ligase Master Mix - 50 rxns **New England Biolabs** **Catalog #**M0367S
- NEBNext Quick Ligation Module - 20 rxns **New England Biolabs** **Catalog #**E6056S
- High Pure Viral Nucleic Acid Kit **Roche** **Catalog #**11858874001
- Agencourt AMPure XP **Beckman Coulter** **Catalog #**A63880
- Agencourt AMPure XP **Beckman Coulter** **Catalog #**A63880
- Agencourt AMPure XP **Beckman Coulter** **Catalog #**A63880
- 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
- Qubit dsDNA BR (Broad Range) assay **Thermo Fisher Scientific** **Catalog #**Q32850
- T7 Endonuclease I - 250 units **New England Biolabs** **Catalog #**M0302S
- Blunt/TA Ligase Master Mix - 50 rxns **New England Biolabs** **Catalog #**M0367S
- Qubit dsDNA BR (Broad Range) assay **Thermo Fisher Scientific** **Catalog #**Q32850

Safety warnings

Follow your facility's regulations and biosafety practices.

Before start

It is recommended to check for the absence of bacterial contamination (e.g., Mycoplasma spp.).

Preparation for virus supernatant

1 Centrifuge the working stock virus to remove debris. 10m

6000 x g, Room temperature, 00:10:00

2 Transfer 180 µL virus supernatant to a 1.5ml screw cap tube.

3 Unwanted DNA and RNA mainly originating from the virus-infected cells are digested using

Micrococcal Nuclease - 320,000 gel units **New England Biolabs Catalog #**M0247S

.

3.1 Total 200 µl reaction 1h

- 179 µL virus supernatant
- 20 µL 10X Micrococcal Nuclease Reaction Buffer
- 1 µL Micrococcal nuclease

Mix by pipetting and spin down.

37 °C water bath 01:00:00

The viral genomic DNA extraction

4 The viral genomic DNA extraction is performed using

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

4.1 Add 200 µL of binding buffer, 4 µL PolyA carrier RNA and 50 µL Proteinase K. 10m

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

72 °C 00:10:00

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

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

8000 x g, Room temperature, 00:01:00 1m

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

4.4 Add 500 µL of inhibitor removal buffer. 1m

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.

4.5 Add 450 µL of wash buffer. 1m

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.

4.6 Add 450 µL of wash buffer. 1m

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 .

4.7 Add 50 µL elution Buffer. 1m

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

Note

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

Amplification of DNA by multiple displacement amplification (MDA)

5 DNA is amplified by MDA using **GenomiPhi V3 Ready-To-Go DNA Amplification Kit - Cytiva Catalog #25-6601-24.**

5.1 Total 20 µl reaction 3m

- 10 µL purified viral genomic DNA

- 10 µL 2X denaturation buffer

Mix by pipetting and spin down.
95 °C 00:03:00
4 °C on ice

5.2 Add 20 µL denatured sample to Ready to go GenomiPhi cake. 4h 10m
30 °C 04:00:00
65 °C 00:10:00

Note

The amplified 20 µL viral genomic DNA can be stored at -80°C.

DNA purification by AMpure XP

6 The DNA is purified using
Agencourt AMPure XP **Beckman Coulter Catalog #**A63880

6.1 Add 10 µL (X0.5 volume) AMPure beads

6.2 Incubate on rotor mixer. 5m
00:05:00 Room temperature

6.3 Spin down and pellet on a magnet. 1m
Wait for 00:01:00 and pipette off the supernatant.

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

6.5 Spin down and pipette off any residual ethanol.

6.6 Resuspend pellet in 40 µL nuclease-free H_2O. 5m
Incubate on a rotor mixer.

00:05:00 Room temperature

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

6.8 Remove and retain 40 µL elute into a new tube.

7 DNA concentration is measured using a Qubit 4 Fluorometer with Qubit dsDNA BR (Broad Range) assay **Thermo Fisher Scientific Catalog #**Q32850 . 2m

- 199 µL 1X working solution
- 1 µL DNA

Mix by vortexing.

Incubate 00:02:00 Room temperature and measure.

Note

Confirm the total amplified DNA to be over 1500 ng.
The DNA can be stored at -30 °C for at least two months.

Note

At this step, whole-genome sequencing using an Illumina next-generation sequencer is also possible. In this case, the T7 endonuclease treatment and any subsequent steps in this protocol are not required. Instead, you should follow Illumina's library preparation protocol. We have confirmed that this works using the Illumina DNA Prep, (M) Tagmentation (Cat#20060060) and the IDT for Illumina DNA/RNA UD Indexes Set A (Cat#20027213).

Similarly, the Oxford Nanopore Rapid Barcoding Kit 24 V14 (SQK-RBK114.24) can also be used. Here, the T7 endonuclease treatment is also not required. From this point, you should proceed by following the Rapid Barcoding Kit protocol, which significantly reduces library preparation time.

T7 endonuclease treatment

8 The amplified DNA by MDA is digested using
T7 Endonuclease I - 250 units **New England Biolabs Catalog #**M0302S .

The following protocol is modified based on the Native barcoding amplicons (with EXP-NBD104, EXPNBD114, and SQK-LSK109) protocol (NBA_9093_v109_revA_12Nov2019) provided by the Oxford Nanopore Technologies website.

8.1 Total 30 µl reaction 30m

- x µL (1.0 µg) DNA
- 3 µL NEBuffer 2
- 1.5 µL T7 endonuclease I
- 25.5-x µL nuclease-free H_2O

Mix by pipetting and spin down.
37 °C 00:30:00

9 The DNA is purified using
Agencourt AMPure XP **Beckman Coulter Catalog #**A63880
Add 15 µL (X0.5 volume) AMPure beads
go to step #6.3
Resuspend pellet in 13 µL nuclease-free H_2O.

DNA repair and end-prep

10 The purified DNA is end-prepped using
NEBNext FFPE DNA Repair Mix - 24 rxns **New England Biolabs Catalog #**M6630S
and
NEBNext Ultra II End Repair/dA-Tailing Module - 24 rxns **New England Biolabs Catalog #**E7546S

10.1 Total 15 µl reaction 35m

- 12 µL DNA
- 0.875 µL NEB Next FFPE DNA repair buffer
- 0.5 µL NEB Next FFPE DNA repair Mix
- 0.875 µL Ultra II end-prep reaction buffer
- 0.75 µL Ultra II end-prep reaction Mix

Mix by pipetting and spin down.

20 °C 00:30:00

65 °C 00:05:00

11 The DNA is purified using

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

Add 15 µL (X1.0 volume) AMPure beads

Resuspend pellet in 10 µL nuclease-free H_2O.

Note

The DNA can be stored at 4°C overnight.

Native barcode ligation

12 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 .

12.1 Total 20 µl reaction 20m

- 8.5 µL DNA
- 1.5 µL native barcode
- 10 µL Blunt/TA ligase master mix

Mix by pipetting and spin down.

Room temperature 00:20:00

12.2 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	2 µl

	EDTA cap colour	Volume per well
	For blue cap EDTA	4 µ

EDTA is added at this step to stop the reaction.

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

14 The DNA is purified using
Agencourt AMPure XP **Beckman Coulter Catalog #**A63880
Add 8.8 or 9.6 µL (X0.4 volume) AMPure beads multiply by the original number of tubes.

14.1 Incubate on rotor mixer. 5m
00:05:00 Room temperature

14.2 Spin down and pellet on a magnet. 1m
Wait for 00:01:00 and pipette off the supernatant.

14.3 Wash twice by 700 µL 70 % ethanol, remove the ethanol using a pipette, and discard.

14.4 Spin down and pipette off any residual ethanol.

14.5 Resuspend the pellet in 15 µL of nuclease-free H_2O. 10m
If the pooled sample volume exceeds 100 µL (i.e., more than 5 samples), resuspend the pellet in x0.2 volume of the pooled sample (e.g., 20 µL when 5 samples are pooled) 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.

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

14.7 Remove and retain the elute into a new tube.

Adaptor ligation and clean-up

20m

15 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

15.1 Total 20 µl reaction 20m

- 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

16 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.

16.1 Add 10 µL (X0.5 volume) AMPure XP reagent and mix by pipetting.
Incubate on a rotor mixer.

00:05:00 Room temperature

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

16.3
- Wash twice by 100 µL Short Fragment Buffer(SFB) and remove the SFB using a pipette and discard.

16.4 Spin down and pipette off any residual SFB.

16.5
- Resuspend pellet in 15 µL Elution Buffer (EB)

5m

00:05:00 Room temperature and tapping occasionally.

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

16.7 Remove retain 15 µL elute into a new tube.

17 DNA concentration is measured using a Qubit 4 Fluorometer with Qubit dsDNA BR (Broad Range) assay **Thermo Fisher Scientific Catalog #**Q32850 . 2m

- 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 length of the major band con¦rmed by electrophoresis after T7 endonuclease treatment. Typically, the fragment lengths are around 2000 base pairs.

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

Sequencing by MinION

19 Sequencing according to the manufacturer's instructions.