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MPXV Sequencing from Wastewater (ONT) V1.0

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Protocol status: Working
We use this protocol and it's working

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Abstract

This protocol is designed to sequence MPXV from wastewater samples using a limited amplicon scheme that targets all MPXV clades, subclades, and lineages.

Materials

Q5® High-Fidelity 2X Master Mix: M0492L, AMPure XP beads: A63882, Qubit High Sensitivity DNA kit: Q32851, D5000 ScreenTape: 5067-5589, D5000 Reagents: 5067-5588, Quick T4 DNA Ligase: 101640-840, NEBNext Ultra End Repair/dA-Tailing Module: E7546S, Invitrogen UltraPure Agarose: 16500500, 1X TAE Buffer (from 50X TAE stock): 1610743, Invitrogen 1 Kb Plus DNA Ladder: 10787018, Thermo Scientific 6X DNA Loading Dye: R0611, Gel electrophoresis system and power source, Thermo Scientific GeneJET Gel Extraction Kit: K0691, Bovine Serum Albumin (BSA) (50 mg/ml): AM2616, Oxford Nanopore Ligation Sequencing Kit V14: SQK-LSK114, Oxford Nanopore Flow Cell R10.4.1: FLO-MIN114, MPXV_v1.0_primer sequences

Viral DNA Amplification

1 Prepare the following master mix per sample (+10%):

	Reagent	Volume in 10 µl reaction
	Q5 Hotstart 2X Master Mix	5
	Primer Pool* (10 µM cumulative; 1 µM cumulative final concentration)	1

∗Primer stocks are in plate format at 100 µM each. To pool, combine equal volume of each primer (100 µM) into a 1.5 mL tube and dilute the pool to 10 µM (cumulative concentration, 0.8 µM in final reaction). Concentration per primer is as follows: (82 oligos) → 10 µM/82 = 0.12 µM per oligo (120 nM) or 0.01 µM in the PCR reaction.
∗Primer scheme available on Andersen Lab Github (https://github.com/andersen-lab/MPXV_wastewater_sequencing)

2 Add 6 µl of PCR master mix to the 96-well PCR reaction plate.

3 Add 4 µl sample to the 96-well PCR plates. Mix by pipetting. Seal plates with foil seals and briefly spin down.

4 Run the following program on thermocycler:
- 1) 98°C for 30 sec
- 2) 95°C for 15 sec
- 3) 60°C for 5 min
- Repeat steps 2-3 for a total of 40 cycles
- 4) 4°C for ∞

5 Check the amplification product on the Tapestation with a D5000 ScreenTape. Expected fragment size ~225-275bp. Spot check a couple samples if running multiple.

5.1 In a PCR tube, add 1 µl of amplified PCR product to 5 µl of sample buffer. Add 1 µl of D5000 ladder to 5 µl of sample buffer.

5.2 Vortex for 1 min at 2000 rpm. Briefly spin before loading on the Tapestation.

Library Construction

6 Combine the tailed primers equimolarly and dilute to 4.1 µM (50 nM for each primer).
*Each primer will be 2 nM per oligo final concentration in solution.

7 Add 90 µl H2O to the amplified DNA **OR** add 9 µl H2O to 1 µl amplified DNA to dilute it 10x. Mix by pipetting.

8 Prepare the following master mix per sample (+10%):

	Reagent	Volume in 10 µl reaction
	Q5 Hotstart 2X Master Mix	5
	Tailed primer pool (50 nM per primer; 5 nM final concentration)*	1
	Nuclease-free water	1

*Primer scheme available on Andersen Lab Github (https://github.com/andersen-lab/MPXV_wastewater_sequencing)

9 Aliquot 7 µl of the PCR master mix in a 96-well PCR reaction plate.

10 Add 1 µl of the unique NB forward primer to each sample (10 µM; 1 µM final concentration).

11 Add 1 µl of the unique NB reverse primer to each sample (10 µM; 1 µM final concentration).

12 Add 1 µl amplified PCR product (10x diluted) to the 96-well reaction plates. Mix by pipetting. Seal plates with foil seals and briefly spin down.

13 Run the following program on thermocycler:
- 1) 95°C for 5 min
- 2) 98°C for 30 sec
- 3) 60°C for 20 min
- 4) 72°C for 2 min
- Repeat steps 2-4 once more
- 5) 98°C for 30 sec
- 6) 65°C for 30 sec
- 7) 72°C for 2 min
- Repeat steps 5-7 14 more times*
- 8) 72°C for 5 min
- 9) 4°C for ∞

*The first part of this PCR ensures that the tailed primers can introduce the adapter overhangs. After that there will be 6 more cycles of PCR. For large batches, this can be lowered (6 total cycles).

Library Clean Up and Quantification

14 Allow AMPure XP beads to equilibrate to room temperature, vortex until homogenous.

15 Add 7 µl beads to 10 µl of the library PCR product. Mix well by pipetting and incubate at room temp for 10 minutes.

16 Place the plate on a magnetic stand and wait until the solution appears clear.

17 Discard supernatant without disturbing the beads.

18 With the plate on the magnet, add 200 µl of 80% EtOH, incubate for 30 seconds, and remove the EtOH wash.
- NOTE: do not resuspend the beads in the wash solution. Just make sure the wash covers the beads stuck to the side of the well.

19 Repeat the previous 80% EtOH wash step and remove as much EtOH as possible.
- NOTE: use a P20 pipette tip to remove all excess EtOH.

20 Leave the plate on the magnet to air dry for 3 min.

21 Remove the plate from the magnet. Add 20 µl of nuclease-free water and mix well by pipetting. Incubate at room temp for 10 minutes.

22 Place the plate on the magnet. When the solution appears clear, remove supernatant without disturbing the beads and place into a new plate.

23 Add 199 µl Qubit HS DNA buffer to a Qubit tube for each sample.

24 Add 190 µl Qubit HS DNA buffer to a Qubit tube for each standard (2).

25 Add 10 µl Qubit HS DNA standard #1 to one standard Qubit tube.

26 Add 10 µl Qubit HS DNA standard #2 to other standard Qubit tube.

27 Transfer 1 µl library from library elution plate to corresponding. Mix well by pipetting.

28 Quantify using the Qubit reader.

29 Check the library product on the Tapestation with a D5000 ScreenTape. Expected fragment size ~400 bp. Spot check a couple samples if running multiple.

Normalize Libraries and Pool

30 Calculate the molarity of the libraries.

31 Pool libraries equimolar to 50ul at 20nM (or highest concentration possible)

End Repair and A-tailing

32 Prepare the following reaction in a PCR tube:

	Reagent	Volume in 60 µl reaction
	NEB Ultra II End Prep Enzyme Buffer Mix	7
	NEB Ultra II End Prep Enzyme Mix	3
	Library Pool	50

33 Run the following program on thermocycler:
- 1) 20°C for 30 min
- 2) 65°C for 30 min
- 3) 4°C for ∞

Gel Clean-up and Purification

34 Make a 1.5% agarose gel with a well large enough to hold 70ul

35 Prepare the sample and controls for running on a gel (for a 10x loading dye):
- Ladder: 9 uL ladder + 1 uL loading dye
- Sample: 60 uL + 6.6 uL loading dye

36 Load the samples and run the gel for about 45 minutes at 110V

37 Excise the band at around 400bp

38 Extract it following the protocol from the gel extraction kit and elute in 30µl of water

Adapter Ligation

39 Dilute A-tailed library pool to 9.0 nM

40 Combine the following in a 1.5 mL Eppendorf tube:

	A	B
	A-tailed library pool (9.0 nM)	30
	Nuclease-free water	30
	Ligation Buffer (LNB)	25
	NEBNext Quick T4 DNA Ligase	10
	Ligation Adapters (LA)	5

41 Mix gently by flicking the tube, and spin down. Incubate the reaction for 10 minutes at room temperature.

Adapter Clean-Up and Quantification

42 Add 0.4x (40 µl) of resuspended AMPure XP beads (AXP) to the reaction and mix by flicking the tube. Incubate for 5 minutes at room temperature.

43 Wash the beads by adding 250 µl Short Fragment Buffer (SFB). Flick the beads to resuspend, spin down, then return the tube to the magnetic rack and allow the beads to pellet. Remove the supernatant using a pipette and discard.

44 Repeat the previous step for a total of 2x washes.

45 Spin down and place the tube back on the magnet. Pipette off any residual supernatant. Allow to dry for ~30 seconds, but do not dry the pellet to the point of cracking.

46 Remove the tube from the magnetic rack and resuspend the pellet in 15 µl Elution Buffer (EB) by pipetting. Spin down and incubate for 10 minutes at room temperature.

47 Place the tube on a magnetic rack. Once the solution clears, elute into a new 1.5 mL Eppendorf tube.

48 Determine DNA concentration of the ligated library pool using Qubit High Sensitivity DNA kit

Priming and Loading Flow Cell

49 Load sample according to ONT manufacturer's instructions