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MPXV Sequencing from Wastewater (Illumina) V1.0 V.1

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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, 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 Barcode i7 primer to each sample (10 µM; 1 µM final concentration).

11 Add 1 µl of the unique Barcode i5 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 Dilute libraries to a 2nM pool.

32 Load sample on the Illumina sequencer according to manufacturer's instructions.