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Influenza Whole Genome Sequencing with Integrated Indexing on Oxford Nanopore Platforms

Forked from Next Generation Sequencing of Influenza Samples for CEIRS

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1 *Works for me* Share

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DISCLAIMER

This protocol is for Research Use Only (RUO).

ABSTRACT

This protocol describes a method for influenza whole-genome sequencing with integrated molecular indexing, which enables a one-step process.

ATTACHMENTS

ms-PCR Template.xlsx

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FORK NOTE

FORK FROM

Forked from Next Generation Sequencing of Influenza Samples for CEIRS, Kenneth Bowden

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GUIDELINES

When processing flu samples, make sure to perform lysis step inside an approved virus hood. Always use PPC and aseptic technique.

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RNA Extraction using MagMAX Viral RNA Isolation Kit

1 Preparation and Stock Solutions for Viral Isolation using MagMAX Viral RNA Isolation kit

On the attached spreadsheet, fill out the 'Sample list" and 'Plate Maps' tab

Go to the 'RNA Isolation Reagents' tab to calculate total reagent amounts.

Lysis Buffer:
Prepare fresh each time a sample is to be extracted. Lysis buffer is one volume of stock solution and one volume of 100% isopropanol. Reagents used for metagenomic sequencing should be dedicated to this particular task, as contaminants are a high risk.

Bead Mix: Magnetic Beads + Lysis / Binding enhancer:
After thorough vortexing of magnetic beads (30s, max speed), and quick mixing of lysis solution, prepare 20uL for each sample by adding one volume of each in a separate eppendorf tube. Store this solution on ice until ready to use.

Wash Buffers:
Prepare by recommended procedures, listed on the bottle. Again, use dedicated alcohol

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bottles for metagenomic sequencing. 300uL additions for each wash step are sufficient when extractions are performed in 1.5mL or 2.0mL eppendorf tubes.

Initial Lysis and Nucleic Acid Binding

a. Add two volumes of lysis buffer to one volume of sample
b. Vortex at moderate speed for 5 minutes to initiate lysis
c. Add 20uL Bead Mix to each tube
d. Vortex tube at low speed for 5 minutes to bind RNA to beads.
e. Perform a quick spin to remove any excess lysis solution from tube cap.
f. Move tubes to magnetic strip for 3 min to capture RNA binding beads.
g. Carefully aspirate supernatant and discard without disturbing the beads
h. Remove tubes from the magnetic strip.

Wash 1

a. Add 300uL of Wash Solution 1 / Isopropanol mixture to each tube.
b. Close tube and mix by inversion for 30s, or vortex at low speed.
c. Move tubes to magnetic strip for 3 min to capture RNA binding beads.
d. Carefully aspirate supernatant and discard without disturbing the beads
e. Remove from magnet and perform a quick spin to remove any excess wash solution from tube cap.
f. Repeat a-e one additional time, for a total of two washes with Wash 1 solution. with Wash 1 solution.

Wash 2

a. Add 300uL of Wash Solution 2 / Ethanol mixture to each tube.
b. Close tube and mix by inversion for 30s, or vortex at low speed.
c. Move tubes to magnetic strip for 3 min to capture RNA binding beads.
d. Carefully aspirate supernatant and discard without disturbing the beads
e. Remove from magnet and perform a quick spin to remove any excess wash solution from tube cap.
f. Repeat a-e one additional time, for a total of two washes with Wash 2 solution.

Elution

a. Move tubes back to magnet for 1m
b. Remove all remaining wash buffer
c. Allow magnetic beads to air dry for 5m with cap open
i. Beads should lose glossy appearance after drying
d. Remove tubes from magnet rack and add 30uL Elution Buffer to each
e. Resuspend beads by pipetting; incubate for 5m
f. Return tubes to magnet rack
g. Remove all 30uL to clean 1.5mL eppendorf tube
i. If sample is to be used immediately (<3h): place tube on wet ice and proceed with downstream processing.

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ii. If sample is to be stored longer term (overnight to 3 weeks): immediately freeze on dry ice and/or place
in -80˚C

ms-PCR

2 Reagents

1. Superscript III High-Fidelity RT-PCR Kit (18080093)
2. Indexed Primer sets (MB TUNI 12 indexed; MB TUNI 13 unindexed) in 10 µM concentrations

A	B	C
PT-00285	MB TUNI 12 ONT BC01 phosphorylated	/5Phos/AAGAAAGTTGTCGGTGTCTTTGTGAGCAAAAGCAGG
PT-00286	MB TUNI 12 ONT BC02 phosphorylated	/5Phos/TCGATTCCGTTTGTAGTCGTCTGTAGCAAAAGCAGG
PT-00287	MB TUNI 12 ONT BC03 phosphorylated	/5Phos/GAGTCTTGTGTCCCAGTTACCAGGAGCAAAAGCAGG
PT-00288	MB TUNI 12 ONT BC04 phosphorylated	/5Phos/TTCGGATTCTATCGTGTTTCCCTAAGCAAAAGCAGG
PT-00289	MB TUNI 12 ONT BC05 phosphorylated	/5Phos/CTTGTCCAGGGTTTGTGTAACCTTAGCAAAAGCAGG
PT-00290	MB TUNI 12 ONT BC06 phosphorylated	/5Phos/TTCTCGCAAAGGCAGAAAGTAGTCAGCAAAAGCAGG
PT-00291	MB TUNI 12 ONT BC07 phosphorylated	/5Phos/GTGTTACCGTGGGAATGAATCCTTAGCAAAAGCAGG
PT-00292	MB TUNI 12 ONT BC08 phosphorylated	/5Phos/TTCAGGGAACAAACCAAGTTACGTAGCAAAAGCAGG
PT-00293	MB TUNI 12 ONT BC09 phosphorylated	/5Phos/AACTAGGCACAGCGAGTCTTGGTTAGCAAAAGCAGG
PT-00294	MB TUNI 12 ONT BC10 phosphorylated	/5Phos/AAGCGTTGAAACCTTTGTCCTCTCAGCAAAAGCAGG
PT-00295	MB TUNI 12 ONT BC11 phosphorylated	/5Phos/GTTTCATCTATCGGAGGGAATGGAAGCAAAAGCAGG
PT-00296	MB TUNI 12 ONT BC12 phosphorylated	/5Phos/CAGGTAGAAAGAAGCAGAATCGGAAGCAAAAGCAGG
PT-00298	MB TUNI 13 phosphorylated	/5Phos/ACGCGTGATCAGTAGAAACAAGG

3. Nuclease-Free Water

Thaw all reagents on ice

Pre-warm thermocycler to 55˚C

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Prepare Master Mix

On the attached spreadsheet, use the 'msPCR' tab to calculate master mix volume

Load the Plate

Add 20 uL of Master Mix to the appropriate wells

Add 5 uL of Sample RNA to appropriate wells

Thermocycler Conditions

Load the plate into the prewarmed thermocycler

Select the following cycling parameters:

55˚C	2 min	
42˚C	60 min	
94˚C	2 min	
94˚C	30 sec	5 cycles
44˚C	30 sec	
68˚C	3.5 min	
94˚C	30 sec	26 cycles
57˚C	30 sec	
68˚C	3.5 min	
68˚C	10 min	
4˚C	hold	

AMPure Clean-Up

3 Equipment/Reagents

1. AMPure XP Bead Mix
2. 70% Ethanol
3. 1x TE Buffer (or Elution Buffer)
4. Magnetic Stand

Protocol

1. Gently shake the Agencourt AMPure XP bottle to resuspend any magnetic beads that may have settled.
2. Add 24 µL (0.8x volume) of resuspended AMPure XP beads to each sample well.
3. Mix reagent and PCR reaction thoroughly by pipetting at least 10 times. Let the mixed sample incubate for 5 minutes at room temperature.

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4. Place the microcentrifuge tube containing the beads on a magnetic stand for 2 minutes. Wait for the solution to clear before proceeding to the next step.
5. Aspirate the cleared solution from the tube and discard.
6. Keep the tube on the magnetic stand, dispense 200 µL of 70% ethanol and incubate for 30 seconds at room temperature. Aspirate out the ethanol and discard. Repeat for a total of two washes. Take care not to disturb the beads while washing.
7. After the last wash, use a p-20 to ensure that all wash buffer is removed from the wells.
8. Air-dry beads for 10 minutes while the tube is on the magnetic stand with the lid open.
9. Take the tube off the magnetic stand, add 30 µL elution buffer (1x TE) and mix by pipetting 10 times.
10. Place the tube on the magnetic stand for 2 minutes to separate the beads, and transfer 30 µL to a new 1.5 mL tube. Avoid transferring any beads.
11. Measure the concentration of the purified DNA with a Nanodrop or Qubit (preferred) and store at −20°C. This procedure typically yields 50–80 ng/ µL of DNA, depending on the amount and quality of the template RNA.

Pooling

4 Pool all samples equi-mass into a single tube, then quantify final product.

Oxford Nanopore Sequencing

5 Proceed with Oxford Nanopore "1D by Ligation" DNA sequencing protocol (Manufacturer-Specific)

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