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Nanopore Influenza A Virus WGS using one step RT/PCR with RBK barcoding

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

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Abstract

A protocol to generate Influenza A Virus sequences from RNA extracts in under 24 hours with less than two hours of hands on time.

Based on:

- King, J., Harder, T., Beer, M., & Pohlmann, A. (2020). Rapid multiplex MinION nanopore sequencing workflow for Influenza A viruses.*BMC Infectious Diseases*,*20*(1). https://doi.org/10.1186/s12879-020-05367-y
- Zhou, B., Donnelly, M. E., Scholes, D. T., st. George, K., Hatta, M., Kawaoka, Y., & Wentworth, D. E. (2009). Single-Reaction Genomic Amplification Accelerates Sequencing and Vaccine Production for Classical and Swine Origin Human Influenza A Viruses.*Journal of Virology*,*83*(19), 10309–10313. https://doi.org/10.1128/jvi.01109-09

Tested with up to 20 RNA extracts from samples per run, alongside:

- Negative control of nuclease-free water
- Positive control of H1
- Positive control of H3
- Positive control of lambda phage, introduced at barcoding stage (i.e. not amplified), as an assessment of barcode crosstalk

Materials

Up to 20 RNA extracts from clinical samples

Consumables

- Zeptometrix H1N1 positive control (A/Puerto Rico/8/1934)
- Zeptometrix H3N2 positive control (A/Hong Kong/2671/19)
- SuperScriptTMIII RT/PlatinumTMTaq Mix (https://www.thermofisher.com/order/catalog/product/12574018)
- 2X Reaction Mix (a buffer containing 0.4 mM of each dNTP, 3.2 mM MgSO4)
- TUNI 12.4: 5' ACG CGT GAT CAG CAA AAG CAG G 3'
- TUNI 12: 5' ACG CGT GAT CAG CAA AAG CAG G 3'
- TUNI 13: 5' ACG CGT GAT CAG CAA AAG CAG G 3'
- SQK-RBK110.96 (https://store.nanoporetech.com/uk/rapid-barcoding-kit-1.html), containing Rapid Barcode plates, AMPure XP Beads, Sequencing Buffer II, Rapid Adapter F, Elution Buffer, Loading Beads II, Loading Solution, Flush Tether, Flush Buffer
- 0.5 ml Eppendorf DNA LoBind tubes
- 2 ml Eppendorf DNA LoBind tubes
- 0.2 ml thin-walled PCR tubes
- Nuclease-free water
- Freshly-prepared 80% ethanol in nuclease-free water
- EXP-CTL001 (https://store.nanoporetech.com/uk/control-expansion.html), containing lambda phage
- Qubit™ dsDNA BR Assay Kit (https://www.thermofisher.com/order/catalog/product/Q32850)

Equipment

- Cold block
- Microplate centrifuge
- PCR stip/Eppendorf centrifuge
- Timer
- Thermal cycler
- Magnetic rack
- Hula mixer (gentle rotator mixer)
- P1000 pipette and tips
- P200 pipette and tips
- P100 pipette and tips
- P20 pipette and tips
- P2 pipette and tips
- Multichannel pipette
- Qubit fluorometer (or equivalent for QC check)
- Flow Cell (R9.4.1)
- GridION Mk1

Troubleshooting

Combined RT/PCR

4h

1 Prepare up to 20 RNA extracts from samples plus:

- Negative control of nuclease-free water
- Positive control of H1N1
- Positive control of H3N2

If samples previously frozen, mix by briefly vortexing and pulse spin to collect liquid. Keep samples on cool block at all times.

2 In the **template-free pre-PCR hood,** prepare the following master mixes in Eppendorf DNA LoBind tubes, mix thoroughly by pipetting, and pulse spin to collect liquid at the bottom of the tube.

Pool A

	Component	Volume	x(n+2)	LOT
	Superscript III	0.5 µL		
	TUNI 12.4/13 (10uM)	1 uL		
	Nuclease Free Water	1.75 µL		
	Reaction Mix x2	6.25 µL		
	Total	9.5 µL		

Pool B

	Component	Volume	x(x+2)	LOT
	Superscript III	0.5 µL		
	Nuclease Free Water	1.75 µL		
	Reaction Mix x2	6.25 µL		
	TUNI 12/13 (10uM)	1 uL		
	Total	9.5 µL		

3 In the **template-free pre-PCR hood**, aliquot 9.5 µL of Pool A master mix into in 0.2 mL 8 well PCR strip-tubes, and 9.5 µL of Pool B into a seperate 0.2 mL 8 well PCR strip tubes.

4 In the **template pre-PCR hood**, transfer 3 µL of sample into the corresponding well of both Pool A and Pool B. Mix by pipetting each well up and down. Spin down.

5 **In the post-PCR lab**, incubate the reaction as follows:

	Step	Temperature	Time	Cycles
	RT step	55	30 minutes	1
	Denaturation	94	2 minutes	1
	Denaturation	94	30 seconds	5
	Annealing	45	30 seconds	5
	Extension	68	3 minutes	5
	Denaturation	94	30 seconds	31
	Annealing	57	30 seconds	31
	Extension	68	3 minutes	31
	Final Extension	68	3 minutes	1
	Hold	4	-	-

Rapid Barcoding

10m

6 Add 5 µL of pool A and 2.5 µL of pool B of each sample to a new strip tube.

7 In another well of the new 0.2ml PCR strip tube, add 0.1 µL of lambda phage to 7.4 µL nuclease free water

8 Transfer 2.5 µL from the Rapid Barcode Plate to the corresponding well of 7.5 µL containing pooled PCR product. Mix by pipetting. Spin down.

9 Incubate the plate: 4m

- 30 °C for 00:02:00

- 80 °C for 00:02:00
- 4 °C hold

Pooling and clean-up

30m

10 Briefly spin down the Barcode reaction product to collect the liquid at the bottom of the wells prior to opening.

11 Pool 10 µL from each sample into a 2ml Eppendorf

12 Vortex pooled samples

13 Resuspend the AMPure XP Beads (AXP, or SPRI) by vortexing

14 To the pooled barcoded sample, add an 0.4 x volume of resuspended AMPure XP Beads (AXP, or SPRI) (e.g. 40 µL of beads to 100 µL of pooled barcode reaction) and mix by pipetting.

15 Incubate on a Hula mixer (rotator mixer) for 00:05:00 at room temperature. 5m

16 Prepare at least 3 mL of fresh 80% ethanol in nuclease-free water.

17 Spin down the sample and pellet on a magnet for 00:03:00. Keep the tube on the magnet, and pipette off the supernatant. 3m

18 Keep the tube on the magnet and wash the beads with 1 mL of freshly-prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.

19 Repeat the previous step.

20 Briefly spin down and place the tube back on the magnet. Pipette off any residual ethanol. Allow to dry for 00:00:30, but do not dry the pellet to the point of cracking. 30s

21 Remove the tube from the magnetic rack and resuspend the pellet by pipetting in 15 µL Nuclease Free water. Incubate for 00:10:00 at room temperature. 10m

22 Pellet the beads on a magnet for 00:03:00 until the eluate is clear and colourless. 3m

23 Remove and retain 15 µL of eluate containing the DNA library into a clean 1.5 ml Eppendorf DNA LoBind tube.

24 Quantify DNA concentration by using the Qubit dsDNA BS Assay Kit.

25 Take forward 1000 ng of library and make up the volume to 11.25 µL Nuclease Free water.

26 Add 0.75 µL of Rapid Adapter F (RAP F) to 11.25 µL of barcoded DNA.

27 Incubate at room temperature on Hula mixer (rotator mixer) for 00:05:00. Then store on ice until ready to load 5m

Priming and loading flow cell 15m

28 Thaw the Sequencing Buffer II (SBII), Loading Beads II (LBII), Flush Tether (FLT) and Flush Buffer (FB) at room temperature before mixing the reagents by vortexing, and spin down the SBII and FLT at room temperature.

29 To prepare the flow cell priming mix, add 30 µL of thawed and mixed Flush Tether (FLT) directly to the tube of thawed and mixed Flush Buffer (FB), and mix by vortexing at room temperature.

30 On the GridION Mk1, place flow cell and perform flow cell check. Proceed if more than >800 pores.

31 Slide the priming port cover clockwise to open the priming port.

32 After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles (a few µl):

- Set a P1000 pipette to 200 µl
- Insert the tip into the priming port
- Turn the wheel until the dial shows 220-230 ul, to draw back 20-30 ul, or until you can see a small volume of buffer entering the pipette tip
- **Note:**Visually check that there is continuous buffer from the priming port across the sensor array.

33 Load 800 µL of the priming mix into the flow cell via the priming port, again by turning wheel of pipette and not by pressing on button, avoiding the introduction of air bubbles. Wait for 5 minutes. During this time, prepare the library for loading by following the steps below.

34 Thoroughly mix the contents of the Loading Beads II (LBII) by pipetting

35 In a new tube, prepare the library for loading as follows:

	Reagent	Volume
	SBII	37.5 uL
	LBII	25.5 uL
	DNA library	12 uL
	Total	75 uL

36 Complete the flow cell priming:

1. Gently lift the SpotON sample port cover to make the SpotON sample port accessible.

2. Load 200 µL of the priming mix into the flow cell via the priming port (**not** the SpotON sample port), avoiding the introduction of air bubbles.

37 Mix the prepared library gently by pipetting up and down just prior to loading.

38 Add 75 µL of sample to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next.

39 Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION device lid.

MinKNOW settings

40 When starting the sequencing run, ensure the following settings are selected

Rapid Barcoding Kit 96 (SQK-RBK110.96)
High accuracy base calling
Enable barcoding
Mid-read barcoding filtering on
Over-ride minimum barcode score 60
Fastq size set to 250 reads

Sequencing performed on MinKNOW version 22.10.7 with guppy_basecaller 6.3.9