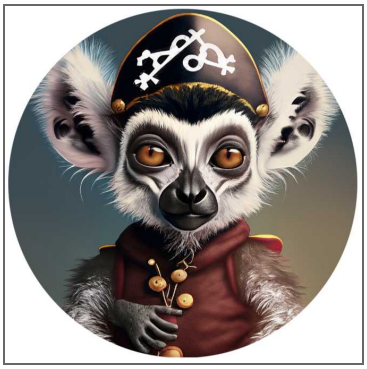

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Integrated Indexing One-Step RT-PCR with NEB LunaScript: Influenza A Virus Sequencing with Native Oxford Nanopore Software Compatibility

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ABSTRACT

This integrated indexing RT-PCR protocol has been optimized for whole genome sequencing of influenza A virus (IAV) using Oxford Nanopore's Ligation Sequencing Kit V14. The updated protocol is an optimization of an early approach used across several laboratories for research purposes, and uses New England Biolabs reagent sets including LunaScript® Multiplex One-Step RT-PCR (#E1555), NEBNext dA-Tailing Module (E6053), and the NEBNext Quick Ligation Module (E6056). Previously demonstrated IAV whole genome sequencing approaches for Oxford Nanopore used Superscript III/Platinum Taq RT-PCR reagents input into the Native Indexing reagent set, which results in a complex, multi-step process that requires more than a full working day to carry out.

The improved integrated indexing protocol can be completed within a working day, and incorporation of the molecular index during first-strand cDNA synthesis in the RT-PCR step reduces the likelihood of sample cross-contamination during handling. Recent improvements to the approach additionally include Oxford Nanopore defined constant regions that are required for full compatibility with default informatics packages. This update now enables native demultiplexing within either Guppy or Dorado, effectively serving as a "molecular patch" where previous implementations required software modifications that were often broken by silent updates to MinKNOW.

As designed, this protocol has potential for extension to other viruses of interest by modifying primer sets. A simplified excel workbook is provided for design of new primer sets, for which the first worksheet requires input of gene-specific primer sets, the second worksheet provides fully constructed primers, and the third can be exported directly for submission to a primer synthesis vendor such as IDT DNA.

ATTACHMENTS

Process Overview.png

MATERIALS

New England Biolabs Components:

	A	B	C	D	E	F
	NEB #	***Component Name***	***Component #***	***Stored at (°C)***	***Amount***	***Concentration***
	E6056	NEBNext Quick Ligation Reaction Buffer	E6058AVIAL	-20	1 x 0.2 ml	5 X
	E6056	Quick T4 DNA Ligase	E6057AVIAL	-20	1 x 0.1 ml	Not Applicable
	E6053	NEBNext dA-Tailing Reaction Buffer	E6055AVIAL	-20	1 x 0.1 ml	10 X
	E6053	Klenow Fragment (3´→ 5´ exo–)	E6054AVIAL	-20	1 x 0.06 ml	Not Applicable
	E1555	LunaScript‰ Multiplex One-Step RT-PCR Enzyme Mix	M1556SVIAL	-20	1 x 0.05 ml	25 X
	E1555	LunaScript‰ Multiplex One-Step RT-PCR Reaction Mix	M1557SVIAL	-20	1 x 0.25 ml	5X
	E1555	Nuclease-free Water	B1502AVIAL	-20	1 x 1.5 ml	Not Applicable

Oxford Nanopore Components:

	A	B	C	D	E	F
	ONT #	***Component Name***	***Component #***	***Stored at (°C)***	***Amount***	***Concentration***
	SQK-LSK114	Ligation Sequencing Kit V14		-20	1 x 0.2 ml	6 Runs
		R10.4.1 Sequencing Flowcell		4		

Other Equipment:

	A

	A
	Component Name
	Qubit
	Tapestation
	Ethanol (molecular biology grade)
	Custom Indices

BEFORE START INSTRUCTIONS

Note: High quality isolated Influenza A RNA must be used for this protocol as it is essential for successful complete genome amplification. There are many options for RNA purification, including solid phase reversible immobilization methods (e.g. spin columns, paramagnetic beads), and Trizol derivatives. Freshly isolated RNA with minimal or no freeze thaw cycles typically ensures the highest success rates for complete genome amplification.

Protocol for Integrated Indexing of Influenza A Virus

1

Prepare the One-Step Reverse Transcriptase Multi-Segment PCR for Influenza A Virus as described below:

	A	B
	Reagent	Volume (uL)
	Purified Viral RNA	5
	5x Buffer (Reaction mix)	10
	Nuclease Free Water (NFW)	31
	Indexed Primer (MBTuni12-NB##)	1
	Unindexed Primer (MBTuni13)	1
	25x Enzyme	2
	Total Volume	50

*Resulting amplicons should range from 700 bp to 2.1kb

** For no template control replace purified viral RNA with NFW

2 Flick the tube or pipet up and down to mix at minimum 10 times followed by a short spin

3 Incubate reaction in thermo-cycler following these steps:

	A	B	C	D
	Step	Temp (C)	Duration (min)	Cycles
	RT Priming	55	2	1
	Reverse Transcription	42	20	1
	RT kill/ PCR Hot Start	98	2	1
	PCR Round One	95	.5	5
		44	.5	
		68	3.5	
	PCR Round Two	95	.5	26
		57	.5	
		68	3.5	
	Final Extension	68	10	1
	Hold	10	∞	∞

3. Library Pooling and Purification

4 Quantify unpurified PCR product using Qubit High Sensitivity DNA reagents

5 Pool indexed PCR products together by equal mass into a single tube for purification

6 Add .6 volumes of AMPure XP to the pooled iiRT-PCR product and mix by finger flicking

> **Note**
>
> Example: 60uL AMPure per 100uL pooled library

7 Incubate for 00:05:00 at room temperature or if available incubate on hula mixer at low speeds 5m
IKA plate shaker at 500 RPM

8 Move to magnetic stand to separate beads, allowing them to sit until solution clears

9 Remove and discard supernatant

10 Add 500 µL of freshly made 80% ethanol

11 Remove tube from magnet and finger flick

12 Repeat steps 8-11 for a total of two washes

13 Allow to dry for 00:05:00 or until bead pellet is no longer shiny 5m

Note

Do not over dry! Pellet will be matte, but will not have cracked.

14 Add 31 µL nuclease-free water and resuspend with gentle pipetting. Incubate for 00:10:00 10m

Note

This 10 minute elution duration is longer than normal Ampure XP, and is intended to maximize the release of larger PCR products.

15 Move to magnetic stand and allow to clear for 00:05:00 5m

16 Transfer purified DNA to new tube

17 Quantify 1 µL of final pooled iiRT-PCR product with high sensitivity qubit reagents

18 (Optional) Following purification of iiRT-PCR products, samples can be analyzed on an agarose gel, Tapestation, or similar platform.

DNA A-Tailing with NEBNext dA-Tailing Module 42m 30s

19 Thaw all NEB consumables on ice and once thawed allow mixture to adjust to room temperature. The 30s pipette the buffer up and down to. Break up the precipitate and then a 00:00:30 vortex.

20 Place 100-200 fmol of barcoded, pooled and purified DNA into a 1.5 Eppendorf tube and adjust volume to

30 µL with nuclease free water

21 Mix in a 0.2 ml PCR plate and then flick and spin down:

	A	B
	Reagents	Volume (uL)
	DNA	30
	NEBNext dA-Tailing. Reaction Buffer (10x)	5
	Klenow Fragment (3’ --> 5’ exo-)	3
	Nuclease Free Water (NFW)	12
	Total Volume	50

22 Incubate plate in thermal cycler at 37 °C for 00:30:00 and then 65 °C for 00:05:0 35m

23 Transfer DNA into 1.5 ml tubes

24 Add 60 µL of AMPure beads to the tube and flick to mix

25 Incubate on hula mixer for 00:05:00 5m

26 Spin down and place on magnet. Once solution is clear pipette off supernatant

27 Add 200 µL of 70% ethanol and remove ethanol without disturbing beads

28 Repeat step 27

29 Take tube off of magnet and spin down, then place back on magnet and remove any residual ethanol

30 Remove tubes from magnet and resuspend pellets in 61 µL of nuclease free water

31 Incubate for 00:02:00 at room temperature 2m

32 Place tubes on magnet and once solution is clear remove supernatant and place in clean 1.5 ml tubes

33 Quantify 1 µL using qubit

Adapter ligation and clean-up 37m

34 Thaw all NEB reagents at room temperature, spin down for 5 seconds, and mix by pipetting. DON'T VORTEX T4 DNA Ligase. Spin down the Ligation Adapter and Quick T4 DNA Ligase, pipette to mix then place on ice. Thaw the Elution Buffer and Short Fragment Buffer at room temp, mix by vortexing, spin down, and then place on ice.

35 Mix by pipetting and spin down the following reaction:

	A	B
	Reagents	Volume (uL)
	Pooled Barcoded Sample	30
	Ligation Adapter (LA)	5
	NEBNext Quick Ligation Reaction Buffer (5X)	10
	Quick T4 DNA Ligase	5
	Total	50

36 Incubate the reaction for 00:20:00 at room temperature 20m

37 Transfer DNA into 1.5 ml tubes

38 Add 20 µL of AMPure beads to each tube and flick to mix

39 Incubate on hula mixer for 00:05:00 5m

40 Spin down and place on magnet. Once solution is clear pipette off supernatant

41 Add 125 µL of Short Fragment Buffer, flick to resuspend, then place back on tube and allow beads to pellet, then remove short fragment buffer without disturbing beads

42 Repeat step 41

43 Take tube off of magnet and spin down, then place back on magnet and remove any residual Buffer

44 Remove tubes from magnet and resuspend pellets in 15 µL of NFW

45 Incubate for 00:10:00 at 37 °C and every 00:02:00 agitate by lightly flicking sample 12m

46 Place tubes on magnet and once solution is clear remove supernatant and place in a clean 1.5 ml tube

> **Note**
>
> This is the final clean library, and should be stored on ice or at 4C until sequencing

Final Library Quantification

47 Quantify 1 µL of the final clean library using a Qubit DNA fluorometer

48 Optional: Run final library on a Tapestation to evaluate average peak size

49 Calculate the final volume required for 200fmol to be sequenced on a MinION flowcell

Note

Tip: Use the NEBioCalculator https://nebiocalculator.neb.com/#!/dsdnaamt. Assuming 1kb product mean, a final loading concentration of 200fmol requires 125ng of library into the flowcell.

50 Proceed to load the Oxford Nanopore flowcell using the most up to date protocols from the vendor.