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NEBNext iiMS Influenza A DNA Library Prep for Oxford Nanopore

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External link: https://github.com/nebiolabs/iiMS_InfluenzaA

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

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Abstract

The NEBNext iiMS Influenza A module for Library Prep (Oxford Nanopore Technologies) includes universal Influenza A primer pairs with indexing tails that allow for generation of indexed full length Influenza A genome segments. The NEBNext iiMS Influenza A Library Prep (ONT) protocol incorporates Oxford Nanopore Native Barcoding sequence during targeted cDNA synthesis and amplification, allowing for the pooling of up to 48 samples directly after this initial reaction. The samples can be demultiplexed using the standard Oxford Nanopore Technologies settings.

Where larger volumes, customized or bulk packaging are required, we encourage consultation with the Customized Solutions team at NEB. Please complete the NEB Custom Contact Form at **www.neb.com/CustomContactForm** to learn more.

Figure 1. Workflow for NEBNext iiMS Influenza A Library Prep for ONT library preparation.

Guidelines

Safe Stop Point: This is a point where you can safely stop the protocol.

Caution: This signifies a step in the protocol that has multiple paths leading to the same end point but is dependent on a user variable, like the amount of input DNA.

(Color): The color in parentheses indicates the cap color of the reagent to be added to a reaction.

Materials

NEBReagents

The volumes described for the primer pairs and LunaScript Multiplex One-Step RT-PCR reagents are sufficient for preparation of up to 48 individual RT-PCR reactions. The NEBNext dA-tailing and NEBNext Ligation modules listed provide enough reagent volumes for up to 20 ONT library preps, as described in this protocol. All reagents should be stored at –20°C. Colored bullets represent the color of the cap of the tube containing the reagent.

From NEB#E1555S

(lilac) LunaScript Multiplex One-Step RT-PCR Enzyme Mix
(lilac) LunaScript Multiplex One-Step RT-PCR Reaction Mix
(white) Nuclease-free Water

From NEB#E6053S

(green) NEBNext dA-Tailing Reaction Buffer
(green) Klenow Fragment (3’ -> 5’ exo–)

From NEB#E6056S

(red) NEBNext Quick Ligation Reaction Buffer
(red) Quick T4 DNA Ligase

From NEB Beta:

NEBNext iiMS Flu A Primer Pairs 1-48

https://github.com/nebiolabs/iiMS_InfluenzaA

Required Materials Not Included

- 80% Ethanol (freshly prepared)
- Nuclease-free Water
- DNA LoBind Tubes (Eppendorf #022431021)
- Any thin wall 200 µl PCR tube, for example TempAssure PCR flex-free 8-stube strips (USA Scientific #1402-4708)
- SPRIselect® ReagentKit (Beckman Coulter®, Inc. #B23317) or AMPure® XP Beads (BeckmanCoulter, Inc. #A63881)
- Magnetic rack/stand (NEB #S1515, Alpaqua® cat. #A001322, or equivalent)
- Vortex
- Thermal cycler
- TapeStation® (Agilent® Technologies, Inc.) and High Sensitivity D5000 DNA ScreenTape
- Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific, Inc.® Q32851)
- Oxford Nanopore Technologies Ligation Sequencing Kit(s); Ligation Sequencing Kit V14 (SQK-LSK114) or Ligation Sequencing Kit XL V14 (SQK-LSK114-XL)

Safety warnings

For hazard information and safety warnings, please refer to the SDS (Safety Data Sheet)

Before start

Please review the important information under the "Guidelines & Warnings" tab before beginning.

Protocol for use with Lunascript Multiplex One-Step RT-PCR Kit (E1555 S/L) with NEBNext iiMS Influenza A Primer Pairs 1-48

1 **Note:**
1) We recommend setting up the RT-PCR reactions in a room (and ideally in a hood) separate from the library construction area to minimize cross-contamination of future reactions.
2) We recommend inputs of ≥ 1,000 copies of the Influenza A viral genome.
3) The presence of carry-over products can interfere with sequencing accuracy, particularly for low copy targets. Therefore, it is important to carry out the appropriate no template control (NTC) reactions to demonstrate that positive reactions are meaningful. We recommend that the NTC reactions are prepared prior to non-NTC samples following the guidelines outlined below.
4) Precipitates may appear in the LunaScript Multiplex One-Step RT-PCR Reaction Mix upon thawing, resuspend completely prior to use by vortexing or pipetting up and down 10 times.

2 Briefly centrifuge the LunaScript Multiplex One-Step RT-PCR Enzyme Mix to collect solution to bottom of the tube, then place on ice.

3 Thaw the LunaScript Multiplex One-Step RT-PCR Reaction Mix, NEBNext iiMS Flu A Primer Pairs 1-48, and the Nuclease-free Water at room temperature, then place on ice. Prior to use, vortex and briefly centrifuge the Lunascipt Multiplex One-Step Reaction Mix and the NEBNext iiMS Flu A Primer Pairs.

4 For no template controls, mix the following components:

	A	B
	COMPONENT	VOLUME
	(white) Nuclease-free Water	14 µl
	(lilac) LunaScript Multiplex One-Step RT-PCR Reaction Mix	5 µl
	(lilac) LunaScript Multiplex One-Step RT-PCR Enzyme Mix	1 µl
	NEBNext iiMS Flu A Primer Pairs 1-48 (2 µM/ primer)	5 µl
	Total Volume	25 µl

Note: Cover/ seal the NTC after mixing.

5 Prepare individual cDNA synthesis and amplification reaction as described below:
For Pool Set A:

	A	B
	COMPONENT	VOLUME
	RNA template*	5 - 14 µl
	(lilac) LunaScript Multiplex One-Step RT-PCR Reaction Mix**	5 µl
	(lilac) LunaScript Multiplex One-Step RT-PCR Enzyme Mix**	1 µl

	A	B
	NEBNext iiMS Flu A Primer Pairs 1-48 (2 µM/ primer)	5 µl
	(white) Nuclease-free Water	to 25 µl
	Total Volume	25 µl

* NEB recommends ≥ 1,000 copies of Influenza A viral genomes as input for high genome coverage.
**A master mix of the LunaScript Multiplex One-Step RT-PCR Reaction Mix, Lunascript Multiplex One-Step RT-PCR Enzyme Mix, and Nuclease-free Water can be prepared and stored at 4°C for up to 8 hours, or at -20°C for 24 hours prior to use.

6 Mix reactions gently by pipetting up and down or by inverting the tubes, then briefly centrifuge to collect solutions to the bottoms of tubes.
Note: We recommend proceeding with the cDNA amplification and library construction in a different area or room.

7 Place the tube in a thermal cycler with the heated lid set to 105°C or on and perform PCR amplification using the following PCR cycling conditions:

CYCLE STEP	TEMP	TIME	CYCLES
Reverse Transcription	55°C*	30 minutes	1
Reverse Transcription Inactivation & Initial Denaturation	98°C	1 minute	1
Denaturation	95°C	15 seconds	5
Annealing	45°C**	30 seconds	
Extension	72°C	3 minutes	
Denaturation	95°C	15 seconds	25
Annealing	65°C**	30 seconds	
Extension	72°C	3 minutes	
Final Extension	72°C	5 minutes	1
Hold	4°C	∞	1

* A 55°C reverse transcription step temperature is optimal for the WarmStart Reverse Transcriptase. To ensure best performance and full WarmStart Reverse Transcriptase activation, avoid using a reverse transcription temperature below 50°C.
** The annealing temperature is primer-mix dependent, 45°C is optimal for the Influenza A homologous regions of the NEBNext iiMS Flu A Primers and 65°C is optimal for the full-length NEBNext iiMS Flu A Primers.

8 Make a cDNA pool following Option A (Step 9) for an equi-volume pool or Option B (Step 10) for an equi-molar pool.

9 Option A: Make an equal volume cDNA pool by adding 10 - 25 µl of each of the indexed amplified cDNA products to a 1.5 ml DNA LoBind tube. Quantify the pool with a TapeStation or Qubit using a 1/10 dilution (1 µl cDNA pool: 9 µl Nuclease-free Water) of the cDNA pool.

10 Option B: Quantify yields for each indexed amplified samples with a TapeStation or Qubit using 1/10 dilutions (1 µl cDNA: 9 µl Nuclease-free Water) of each cDNA sample. Make an equal molar cDNA pool by adding up to 20 µM of each of the indexed amplified cDNA products into a 1.5 ml DNA LoBind Tube.

11 **Safe Stop Point: Samples can be stored at 4°C overnight or –20°C for long term storage if they are not used immediately.**

Cleanup of pooled indexed cDNA amplicons

12 **Notes:**
1) Use the pooled indexed cDNA samples from Step 9 or 10; a pool volume of 120 - 480 µl is recommended for bead cleanup. Any remaining cDNA can be stored long-term at -20°C.
2) The volumes of AMPure XP Beads described here are for use with the sample contained in the exact buffer at this step. Allow the beads to warm to room temperature for at least 30 minutes before use. These volumes may not work properly for a cleanup at a different step in the workflow. SPRIselect beads may also be used.

13 Vortex AMPure XP Beads to resuspend.

14 Add 0.6X resuspended beads to pooled barcoded samples from Step 8 (For example, if total pool volume is 480 µl, then add 288 µl of resuspended AMPure XP Beads). Mix well by flicking the tube or pipetting up and down 10 times, do not vortex. If necessary, perform a quick spin for 1 second to collect all liquid from the sides of the tube.

15 Incubate samples on bench top for 00:05:00 at room temperature. 5m

16 Place the tube on a 1.5 ml magnetic stand (such as NEB #S1506) to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube before placing on the magnetic stand.

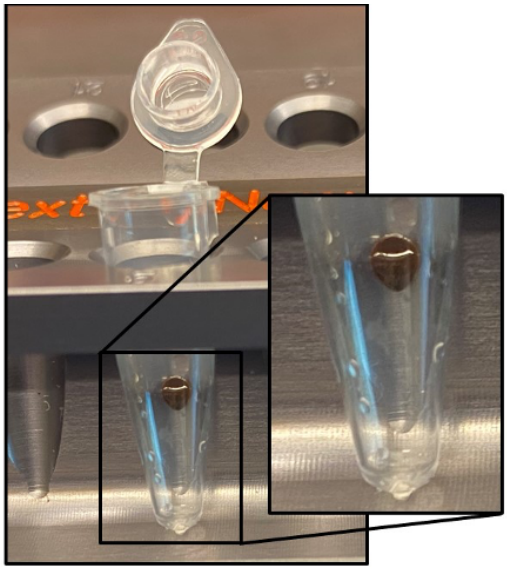
17 After 3 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets (Caution: do not discard beads).

18 Wash the beads by adding 200 to 500 µl of 80% freshly prepared ethanol to the tube while on the magnetic stand. Incubate at room temperature for 30 seconds and then carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets.

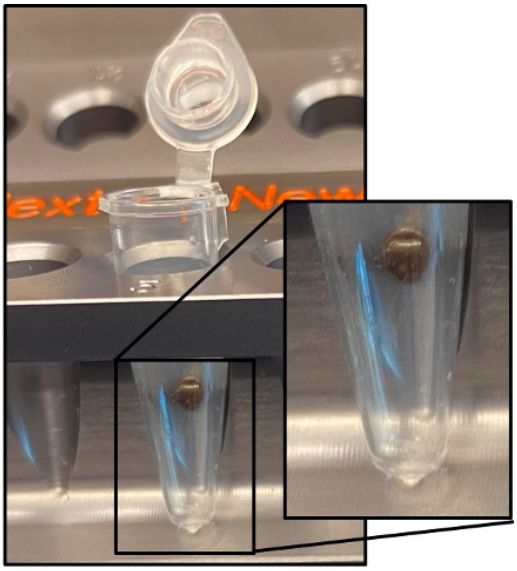
19 Repeat the wash once to make it a total of 2 washes.

20 Perform a quick spin and place the sample tube on the magnetic stand, remove any residual ethanol.

21 Air dry the beads for 30 seconds while the tube is on the magnetic stand with the lid open.
Caution: Do not over-dry the beads. This may result in lower recovery of DNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack, they are too dry.



After ethanol is removed the beads will be shiny and droplets of ethanol will be on the inside of the tube



When the beads are ready to elute visible droplets are gone and the beads are still dark brown and look a little matte

22 Remove the tube from the magnetic stand. Elute the DNA target from the beads by adding 33 µl of Nuclease-free Water

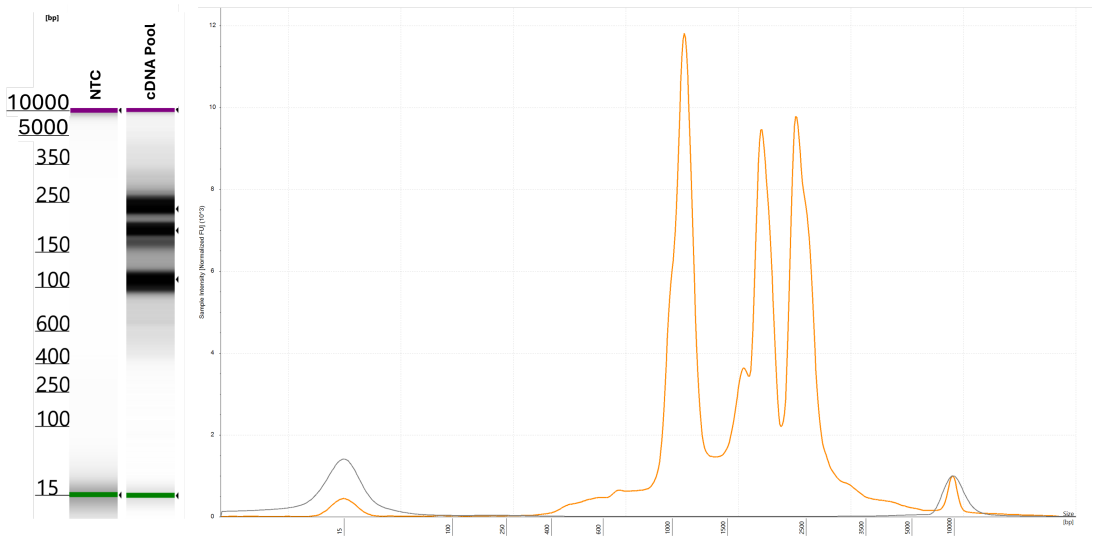
23 Resuspend the pellet by flicking the tube or pipetting up and down 10 times to mix. Incubate for at least 2 minutes at room temperature. If necessary, quickly spin the sample for 1 second to collect the liquid from the sides of the tube before placing back on the magnetic stand.

24 Place the tube on the magnetic stand. After 2 minutes (or when the solution is clear), transfer 32 µl to a PCR tube.
Assess the concentration of the purified barcoded DNA sample. We recommend using a Qubit fluorometer for accurate concentration assessment and TapeStation for cDNA size

confirmation. Nanodrop is NOT recommended since it may overestimate the DNA concentration. Use 1 µl of the eluant to make a 1/10 dilution (1 µl eluant : 9 µl Nuclease-free Water) of the pool for the Qubit fluorometer and TapeStation analyses.
Safe Stop Point: Samples can be stored long term at –20°C if they are not used immediately.

25 **Note:**
The cleaned-up pooled cDNA amplicons may be run on a TapeStation to confirm 900 – 2,500 bp amplicon sizes. To run on a TapeStation, dilute 1 µl of the cleaned-up pooled amplicons 10-fold with Nuclease-free Water and run 2 µl on a High Sensitivity D5000 DNA ScreenTape. (See Figure 1.1 below for example of amplicon size profile).

Figure 1.1: Examples of amplicons prepared from pooled samples for 10,000 genome copies of Influenza A or non-template control.



Diluted cleaned pooled indexed cDNA made from either non-template controls (NTC) or Influenza samples with 10,000 copies of Influenza gRNA each. Pool run on TapeStation HSD5000 ScreenTape.

NEBNext A-tailing

26 Add the following components to a sterile nuclease-free tube **on ice:**

	A	B
	COMPONENT	VOLUME
	Cleaned pooled indexed cDNA from Step 24*	30 µl
	(green) NEBNext dA-Tailing Reaction Buffer	3 µl
	(green) Klenow Fragment (3' -> 5' exo-)	2 µl

	A	B
	Total Volume	35 µl

***Note: NEB recommends cDNA input of 50 - 150 ng for this A-tailing reaction.**

27 Flick the tube or pipette up and down 10 times to mix the solution. Perform a quick spin to collect all liquid from the sides of the tube. A small number of bubbles in the reaction will not inhibit performance.

28 Place in a thermal cycler, with the heated lid set to ≥ 75°C or on, and run the following program: 35m

00:30:00 at 37°C

00:05:00 at 65°C

Hold at 4°C

Proceed Immediately to Adapter Ligation in Step 29.

Adaptor Ligation

29 Add the following components directly to A-tailed cDNA:

	A	B
	COMPONENT	VOLUME
	A-tailed cDNA (Step 28)	35 µl
	(red) NEBNext Quick Ligation Reaction Buffer*	10 µl
	Ligation Adaptor**	5 µl
	(red) Quick T4 DNA Ligase	5 µl
	Total Volume	55 µl

* Mix the NEBNext Quick Ligation Reaction Buffer by pipetting up and down several times prior to adding to the reaction.

** Ligation Adapter is provided in Oxford Nanopore Technologies Ligation Sequencing Kit(s); Ligation Sequencing Kit V14 (SQK-LSK114) or Ligation Sequencing Kit XL V14 (SQK-LSK114-XL).

30 Flick the tube to mix solution. Perform a quick spin for 1 second to collect all liquid from the sides of the tube.

(Caution: The NEBNext Quick Ligation Reaction Buffer is viscous. Care should be taken to ensure adequate mixing of the ligation reaction, as incomplete mixing will result in reduced ligation efficiency. The presence of a small amount of bubbles will not interfere with performance).

31 Incubate at 25°C or room temperature for 20 minutes and proceed to cleanup of Adapter-ligated DNA in Step 32.

Cleanup of Adapter Ligated DNA

15m

32 **Note: The volumes of AMPure XP Beads described here are for use with the sample contained in the exact buffer at this step. Allow the beads to warm to room temperature for at least 30 minutes before use. These volumes may not work properly for a cleanup at a different step in the workflow. SPRIselect beads may also be used**

33 Vortex AMPure XP Beads to resuspend.

34 Add 27.5 µl (0.5X) of resuspended beads to the ligation mix. Mix well by flicking the tube followed by a quick spin for 1 second.

35 Incubate samples for 00:05:00 at room temperature. 5m

36 Place the tube on an appropriate magnetic stand to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic stand.

37 After 3 minutes (or when the solution is clear), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets **(Caution: do not discard the beads).**

38 Wash the beads by adding 125 µl of Short Fragment Buffer (SFB) provided in the Ligation Barcoding Kit from Oxford Nanopore Technologies. Flick the tube to resuspend pellet. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand. Place the tube on an appropriate magnetic stand.

39 Wait for 3 minutes (or until the solution is clear) to separate the beads from the supernatant. Remove the supernatant.

40 Repeat steps 38 and 39 once for a total of 2 washes. Remove all visible liquid after the second wash. If necessary, briefly spin the tube/ plate, place back on the magnet and remove traces of SFB with a p10 pipette tip. Once the SFB has been removed, proceed to Step 41.
Caution: Do not over-dry the beads. This may result in lower recovery of DNA target. If beads have turned light brown and start to crack, they are too dry.

41 Remove the tube from the magnetic stand. Elute the DNA target from the beads by adding 15 µl of Elution Buffer (EB) provided in the Ligation Barcoding Kit from Oxford Nanopore Technologies.

42 Resuspend the pellet well in EB buffer by flicking. Incubate for 00:05:00 at room temperature. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand. 5m

43 Place the tube/ plate on the magnetic stand. After 3 minutes (or when the solution is clear), transfer 15 µl to a new DNA LoBind tube.

44 Use Qubit to quantify 1 µl of DNA sample. Follow Oxford Nanopore Technologies Ligation Sequencing Kit Protocol for preparing the flow cell and DNA library sequencing mix using 30 ng - 60 ng adapter-ligated cDNA sample (Step 43).
Note: After normalizing the DNA to 30 ng - 60 ng, if the volume is less than 12 µl, then bring up the sample volume to 12 µl with EB.

Protocol references

Peter Thielen 2022. Influenza Whole Genome Sequencing with Integrated Indexing on Oxford Nanopore Platforms. **protocols.io**https://dx.doi.org/10.17504/protocols.io.kxygxm7yzl8j/v1