INSTRUCTION MANUAL

NEBNext® Flu A Integrated Indexing Primer Module

NEB #E3436S

48 reactions

Version 1.0_10/25

Table of Contents

The NEBNext Flu A Integrated Indexing Primer Module Includes

The volumes provided are sufficient for preparation of up to 48 RT-PCR reactions (NEB #E3436S).
All reagents should be stored at –20°C.

- (Plate) NEBNext Flu A Integrated Indexing Primers (Set 1)

Required Materials Not Included

- LunaScript® Multiplex One-Step RT-PCR Kit (NEB #E1555)
- NEBNext dA-Tailing Module (NEB #E6053)
- NEBNext Quick Ligation Module (NEB #E6056)
- Oxford Nanopore Technologies Ligation Sequencing Kit(s):
  Ligation Sequencing Kit V14 (SQK-LSK114) or Ligation Sequencing Kit XL V14 (SQK-LSK114-XL)
  Note: The SQK-LSK114 kit includes AMPure® XP Beads; additional AMPure XP Beads may be required for this workflow, depending on cleanup conditions.
- SPRIselect™ Reagent Kit (Beckman Coulter®, Inc. #B23317) or AMPure XP Beads (Beckman Coulter, Inc. #A63881)
- 80% Ethanol (freshly prepared)
- Nuclease-free Water (NEB #B1500)
- DNA LoBind Tubes (Eppendorf® #022431021)
- DNase-, RNase-free PCR strip tubes (e.g., TempAssure PCR flex-free 8-tube strips USA Scientific #1402-4708)
- Magnetic racks (e.g., NEB #S1506S and NEB #S1515S), magnetic plate (Alpaqua® #A001322), or equivalent
- Vortex mixer
- Thermal cycler
- Microcentrifuge
- Plate centrifuge
- Bioanalyzer® or TapeStation® (Agilent® Technologies, Inc.) and associated reagents and consumables
- Qubit® or Lunatic® (Unchained Labs®) and associated consumables

Overview

The NEBNext Flu A Integrated Indexing Primer Module includes universal Influenza A primer pairs with indexing tails that allow for integrated indexing multi-segment (iiMS) RT-PCR, compatible with the Oxford Nanopore Native Barcoding workflow. The NEBNext Flu A Integrated Indexing Primer Module is intended for use in library preparation upstream of Influenza A sequencing on Oxford Nanopore Technology platforms. The universal Influenza A targeting aspect of these primers reduces the impact of variants on amplification efficiency when identifying and sequencing various Influenza A strains (e.g., H3N2, H1N1, and H5N1) from various host sources (e.g., Human, Avian, Bovine, etc.).

These primers also incorporate Oxford Nanopore Native Barcoding sequences during targeted cDNA synthesis and amplification, allowing for the pooling of up to 48 samples directly after RT-PCR. Primer sequence information can be found at: https://github.com/nebiolabs/NEBNextFluA.

Note: The NEBNext Flu A Integrated Indexing Primer Module contains only primers; all other required reagents must be purchased separately.

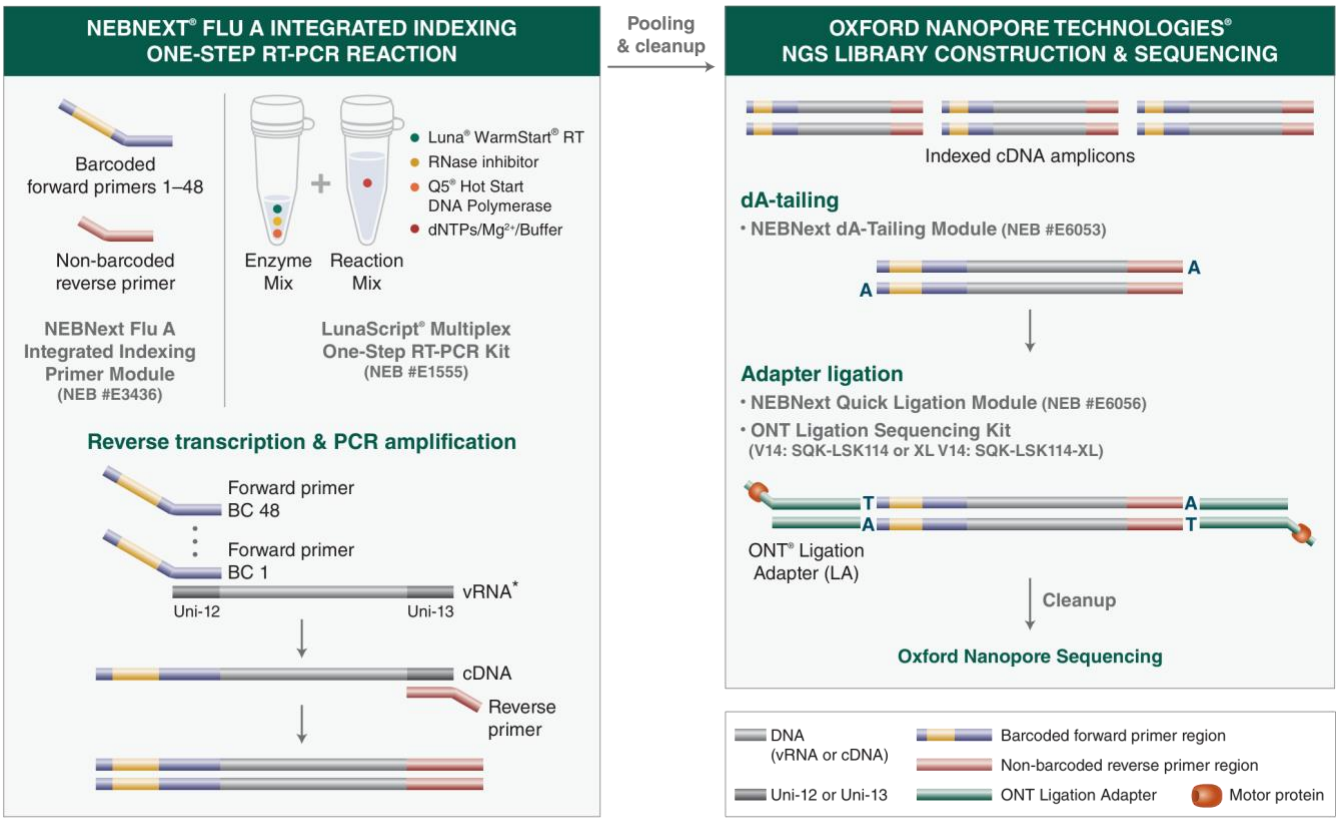
For Oxford Nanopore Technologies sequencing, the NEBNext Flu A Integrated Indexing Primer Module is meant to be used in conjunction with the LunaScript Multiplex One-Step RT-PCR Kit, NEBNext dA-Tailing Module, NEBNext Quick Ligation Module and ONT® Library Prep Reagents, allowing for whole-genome targeted amplification of Flu A and rapid conversion of amplified cDNA into high-quality libraries for sequencing on Oxford Nanopore Technologies platforms.

All module components pass rigorous quality control standards. Module components are also functionally validated via amplicon generation, followed by the construction and sequencing of indexed libraries on an Oxford Nanopore Technologies sequencing platform.

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.

Please refer to the NEBNext Flu A Integrated Indexing Primer Module product listing on NEB.com for FAQs.

Figure 1. Workflow demonstrating the use of NEBNext Flu A Integrated Indexing Primer Module for ONT sequencing



* Recommended inputs of ≥ 1,000 intact copies of the Influenza A viral genome

Experimental Considerations

- Sequence information for NEBNext Flu A Integrated Indexing Primers can be found at: https://github.com/nebiolabs/NEBNextFluA.
- When following the RT-PCR reaction protocol in Section 1, the NEBNext Flu A Integrated Indexing Primer Module contains enough primers for 48 individual integrated indexing RT-PCR reactions when paired with the LunaScript Multiplex One-Step RT-PCR Kit (NEB #E1555).
- We recommend setting up the one-step RT-PCR reaction in a room (and ideally in a hood) separate from the library construction area to minimize cross-contamination of future reactions.
- Inputs of ≥ 1,000 intact copies of the Influenza A viral genome are recommended. The use of lower input amounts or more degraded viral gRNA may result in lower yields and reduced genome coverage.
- When following the protocol for Oxford Nanopore Technologies library preparation in Section 2, pool at least 4 samples per library prep to meet their input and plexity recommendations.
- The NEBNext dA-Tailing Module (NEB #E6053) and NEBNext Quick Ligation Module (NEB #E6056) recommended for the library prep protocol in Section 2 contain sufficient reagents for the preparation of up to 20 Oxford Nanopore Technologies pooled sample libraries.
- To have NEBNext Flu A Integrated Indexing libraries automatically demultiplexed on ONT platforms, select the Native Barcoding Sequencing Kit 96 (SQK-NBD-114-96) demultiplexing option when configuring the sequencing run, despite utilizing the Ligation Sequencing Kit V14 (SQK-LSK114) during library preparation.

Section 1
Indexed Influenza A cDNA Synthesis and Amplification

Symbols

This is a point where you can safely stop the protocol.

This caution sign 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.

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Colored bullets indicate the cap color of the reagent to be added to a reaction.

1.1. One-step RT-PCR with NEBNext Flu A Integrated Indexing Primers

Notes:

(1) We recommend setting up the one-step RT-PCR reaction in a room (and ideally in a hood) separate from library construction area to minimize cross-contamination of future reactions.

(2) Inputs of ≥ 1,000 intact copies of the Influenza A viral genome are recommended. The use of lower input amounts or more degraded viral gRNA may result in lower yields and reduced genome coverage.

(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. NTC reactions should be 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 pipette mixing 10 times.

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

1.1.2. Thaw the LunaScript Multiplex One-Step RT-PCR Reaction Mix, NEBNext Flu A Integrated Indexing Primers (Set 1), and the nuclease-free water at room temperature, then place on ice. Prior to use, vortex and briefly centrifuge the Reaction Mix and Primers.

1.1.3. For no template controls, mix the following components:

COMPONENT	VOLUME
○ (white) Nuclease-free Water	14 µl
○ (white) LunaScript Multiplex One-Step RT-PCR Reaction Mix*	5 µl
○ (white) LunaScript Multiplex One-Step RT-PCR Enzyme Mix*	1 µl
NEBNext Flu A Integrated Indexing Primers (2 µM/primer)**	5 µl
Total Volume	**25 µl**

* A master mix of the RT-PCR Reaction Mix, RT-PCR Enzyme Mix, and nuclease-free water can be prepared and stored at 4°C for up to 8 hours, or -20°C for 24 hours prior to use.

** Each sample to be pooled for sequencing must receive a unique Flu A Integrated Indexing Primer input from a single-use well of the NEBNext Flu A Integrated Indexing Primers (Set 1) plate.

1.1.4. Prepare the amplification reaction as described below:

COMPONENT	VOLUME
RNA template*	5–14 µl
○ (white) LunaScript Multiplex One-Step RT-PCR Reaction Mix**	5 µl
○ (white) LunaScript Multiplex One-Step RT-PCR Enzyme Mix**	1 µl
NEBNext Flu A Integrated Indexing Primers (2 µM/primer)***	5 µl
○ (white) Nuclease-free Water	to 25 µl
Total Volume	**25 µl**

* For achieving high genome coverage, use an input of ≥ 1,000 copies of intact Influenza A viral genomes.

** A master mix of the RT-PCR Reaction Mix, RT-PCR Enzyme Mix, and nuclease-free water can be prepared and stored at 4°C for up to 8 hours, or -20°C for 24 hours prior to use.

*** Each sample to be pooled for sequencing must receive a unique Flu A Integrated Indexing Primer input from a single-use well of the NEBNext Flu A Integrated Indexing Primers (Set 1) plate.

1.1.5. Mix reactions gently by pipetting up and down or by inverting the tubes ten times, then briefly centrifuge to collect solution to the bottoms of tubes.

Note: We recommend proceeding with the cDNA amplification and library construction in a different area or room to minimize amplicon contamination of RT-PCR reactions.

1.1.6. 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
RT 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 RT step temperature is optimal for Luna WarmStart Reverse Transcriptase. To ensure best performance and full WarmStart activation, avoid using an RT temperature below 50°C.

** The annealing temperature is primer-mix dependent, 45°C is optimal for the Influenza A homologous regions of the Flu A Integrated Indexing Primers and 65°C is optimal for the full-length Flu A Integrated Indexing Primers.

1.1.7. Optional, but recommended while optimizing this workflow for your specific samples: Confirm cDNA synthesis and amplification success by running 1/10 dilutions (1 µl cDNA: 9 µl nuclease-free water) of each indexed amplified cDNA sample on a TapeStation D5000 HS ScreenTape®.

1.1.8. Pool 10–25 µl of each of the indexed amplified cDNA products into a 1.5 ml DNA LoBind Tube.

Note: Use 25 µl per sample if there are fewer than 20 samples to be pooled to ensure sufficient volume and DNA input for the subsequent pool cleanup and Oxford Nanopore Technologies library preparation. 10 µl per sample can be used if there are more than 20 samples to be pooled. Excess cDNA can be stored long-term at -20°C.

Samples can be stored at 4°C overnight or –20°C for long-term storage if they are not used immediately.

1.2. Cleanup of pooled indexed cDNA amplicons

Notes:

(1) Use the pooled indexed cDNA products from Step 1.1.8.; a pool volume of 100–480 µl is recommended for bead cleanup.

(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.

1.2.1. Vortex AMPure XP Beads provided in the Ligation Barcoding Kit from Oxford Nanopore Technologies to resuspend.

1.2.2. Add 0.6X resuspended beads to pooled barcoded samples from Step 1.1.8. (For example, if the 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. Perform a quick spin for 1 second to collect all liquid from the sides of the tube.

1.2.3. Incubate samples on bench top for 5 minutes at room temperature.

1.2.4. 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 or plate wells before placing on the magnetic stand.

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

1.2.6. Wash the beads by adding 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.

1.2.7. Repeat step 1.2.6. once to make a total of two washes.

1.2.8. 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.

1.2.9. Remove the tube from the magnetic stand. Elute the DNA target from the beads by adding 33 µl of nuclease-free water.

1.2.10. 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 it back on the magnetic stand.

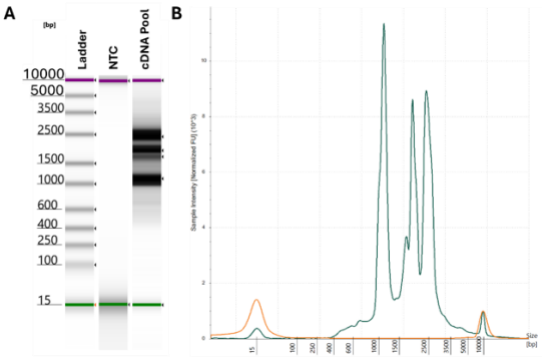
1.2.11. Place the tube on the magnetic stand. When clear (after approximately 2 minutes), transfer 32 µl to a PCR tube.

1.2.12. Assess the concentration of the purified barcoded DNA sample. We recommend using Qubit dsDNA HS reagents for accurate concentration assessment and D5000 HS TapeStation reagents for cDNA size confirmation. Use 1 µl of the eluant from Step 1.2.11. to make a 1/10 dilution of the pool for the Qubit fluorometer and TapeStation analyses.

Samples can be placed at –20°C for long-term storage if they are not used immediately.

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 the cleaned-up pooled amplicons with nuclease-free water and run on a High Sensitivity D5000 DNA ScreenTape (see Figure 1.1. for example of amplicon size profile).

Figure 1.1: Examples of amplicons prepared from pooled samples for 10,000 genome copies of Influenza



1/10th dilution of pooled indexed cDNA after cleanup. cDNA amplicons were made from either non-template controls (NTC) or influenza samples with 10,000 copies of Influenza gRNA each. Pool was run on TapeStation HSD5000 ScreenTape.

Section 2
Influenza A Library Preparation for Oxford Nanopore Technologies

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Colored bullets indicate the cap color of the reagent to be added to a reaction.

2.1. NEBNext A-tailing

2.1.1. Thaw the NEBNext dA-Tailing Reaction Buffer on ice. Prior to use, vortex and briefly centrifuge the Reaction Buffer.

2.1.2. Briefly centrifuge the Klenow Fragment to collect the solution to bottom of the tube, then place on ice.

2.1.3. Add the following components to a sterile nuclease-free tube on ice:

COMPONENT	VOLUME
50–150 ng of cleaned pooled indexed cDNA from Step 1.2.11.*	30 µl
◦ (yellow) NEBNext dA-Tailing Reaction Buffer	3 µl
◦ (yellow) Klenow Fragment (3′→5′ exo)	2 µl
Total Volume	**35 µl**

* For highly concentrated cDNA pools, dilute the pool with nuclease-free water to 150 ng per 30 µl.

2.1.4 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.

2.1.5. Place in a thermal cycler, with the heated lid set to ≥ 75°C, or on, and run the following program:
30 minutes at 37°C
5 minutes at 65°C
Hold at 4°C

Proceed immediately to Adapter Ligation in Section 2.2.

2.2. Oxford Nanopore Technologies Adapter Ligation

2.2.1. Thaw the NEBNext Quick Ligation Reaction Buffer on ice. Prior to use, vortex and briefly centrifuge the Reaction Buffer.

2.2.2 Briefly centrifuge the Quick T4 DNA Ligase and Ligation Adapter to collect the solution to bottom of the tubes, then place on ice.

2.2.3 Add the following components to the A-tailed sample tube:

COMPONENT	VOLUME
A-tailed cDNA (step 2.1.5.)	35 µl
Ligation Adapter*	5 µl
• (red) NEBNext Quick Ligation Reaction Buffer**	10 µl
• (red) Quick T4 DNA Ligase**	5 µl
Total Volume	**55 µl**

* 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).

** A master mix of the NEBNext Quick Ligation Reaction Buffer and Quick T4 DNA ligase can be prepared and stored at 4°C for up to 8 hours prior to use.

Do not premix the Ligation Adapter and Ligase prior to use in the Adapter Ligation Step.

2.2.4 Flick the tube to mix the solution. Perform a quick spin for 1 second to collect all liquid from the sides of the tube.
(Caution: The NEBNext Quick Ligation 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 number of bubbles will not interfere with performance).

2.2.5. Incubate at 25°C for 20 minutes.

Proceed immediately to Cleanup of Adapter-ligated DNA in Section 2.3.

2.3. Cleanup of Adapter-Ligated DNA

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.

2.3.1. Vortex AMPure XP Beads provided in the Ligation Barcoding Kit from Oxford Nanopore Technologies to resuspend.

2.3.2. Add 27.5 µl (0.5X) resuspended beads to the ligation mix. Mix well by flicking the tube 10 times, followed by a quick spin for 1 second.

2.3.3. Incubate samples for 5 minutes at room temperature.

2.3.4. 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.

2.3.5. When clear (after approximately 3 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets (Caution: do not discard the beads).

2.3.6. 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 before placing back on the magnetic stand. Place the tube on an appropriate magnetic stand.

2.3.7. To separate the beads from the supernatant, wait for the solution to clear (approximately 3 minutes). Remove the supernatant.

2.3.8. Repeat steps 2.3.6. and 2.3.7. once for a total of two washes. Be sure to remove all visible liquid after the second wash.

2.3.9. Briefly spin the tube/plate, place back on the magnet and remove traces of SFB with a p10 pipette tip.

2.3.10. 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.

2.3.11. Resuspend the pellet well in EB buffer by flicking. Incubate for 10 minutes 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.

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

2.3.13. Use Qubit to quantify 1 µl DNA sample. Follow Oxford Nanopore Technologies Ligation Sequencing Kit Protocol for preparing the flow cell and DNA library sequencing mix using 50 ng adapter-ligated cDNA sample (Step 2.3.12.).

Notes:

(1) After normalizing the DNA to 50 ng, if the volume is less than 12 µl, top off the sample volume to 12 µl with EB.

(2) The 50 ng loading amount above assumes an average library size of ~1.5 kb. The NEB dsDNA Mass to/from Moles Convertor available at https://nebiocalculator.neb.com/ can be used to more accurately determine library loading amount based on the average library size of your samples.

(3) Follow ONT SQK-NBD114 protocols and recommendations for MinION® or PromethION® Flow Cell priming and loading.

Kit Components

NEB #E3436S Table of Components

NEB #	DESCRIPTION	VOLUME
E3436AVIAL	NEBNext Flu A Integrated Indexing Primers (Set 1)	5 µl/well

Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	10/25