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# Barcoded Primers: Influenza A Whole Genome Amplification for Nanopore Sequencing

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NGS Optimization



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**Protocol status:** Working

**We use this protocol and it's working**

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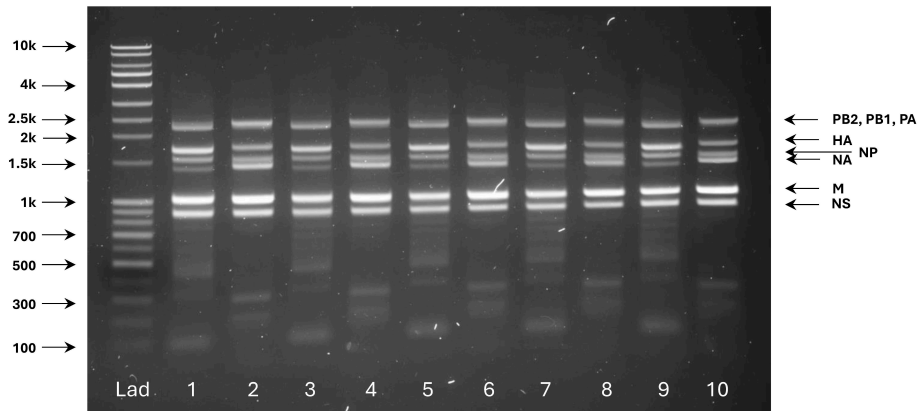
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## Abstract

This protocol describes an optimized method for the simultaneous amplification and barcoding of all eight influenza A segments using Barcoded Primers with either the SuperScript IV One-Step RT-PCR Kit (Invitrogen) or the LunaScript Multiplex One-Step RT-PCR Kit (NEB). The purified barcoded amplicons can be used for Oxford Nanopore library preparation with the Ligation Sequencing Kit for multiplexed whole-genome sequencing of influenza A virus genomes.



**Fig. 1. Full length amplification of all Influenza A segments:** six fragments of approximately 2.3 kb (PB2, PB1 and PA), 1.8 kb (HA), 1.6 kb (NP), 1.5 kb (NA), 1.1 kb (M) and 0.9kb (NS) in length.

## Materials & Consumables

### 1 Materials

- Barcoded primers:  Barcoded Flu A Primers.xlsx 12KB
- 5 µl of Influenza A RNA

### 2 Consumables

SuperScript IV One-Step RT-PCR System (Invitrogen, cat. number 12594100 or 12594025) or LunaScript Multiplex One-Step RT-PCR Kit (NEB, cat. number E1555S or E1555L);

Select-a-Size DNA Clean & Concentrator (Zymo Research, cat. number D4080)

## Primer Mix

- 3 First, create a master  100 micromolar (µM) stock solution for each primer and then dilute it to a  20 micromolar (µM) working solution mix for each barcode. This reduces the number of freeze/thaw cycles that the primer stock goes through and reduces the chances of contamination.

### 4 Preparation of Primer Stock Solutions (100 µM)

10s

#### Note

The microliters of water required to create a  100 micromolar (µM) solution is 10x the nanomoles of lyophilized primer (i.e., if 26.5 nmol is noted on side of tube, a 100 µM primer stock solution is created by adding  265 µL of water or TE buffer to stock tube). The original primer tubes are used for a 100 µM stock.

- Find the oligo yield information in nmol on your tube label
- Multiply this number by 10. The resulting product is the amount of water or TE buffer needed (in µL)
- Reconstitute lyophilized primers in required volume of molecular grade water (i.e., RNase/DNase free) or TE buffer
- Once water has been added, vortex for at least  00:00:10 to ensure the lyophilized primers are fully dissolved and store at  -20 °C to use as needed

### 5 Preparation of Primer Working Solutions (20 µM)

In a clean template-free pre-PCR hood, prepare working solution of the Primer Mix per each barcode:

- Allow stock solution to thaw completely at Room temperature
- Vortex the stock solution for at least 00:00:10 to ensure homogenization before adding it to the working solution
- Prepare a 100 20 micromolar ( $\mu\text{M}$ ) working solution by combining stock solutions (100  $\mu\text{M}$ ) of Barcoded F1/F2/R primers in 0.35:0.65:1 ratio for each barcode:

|  | COMPONENT           | CONCENTRATION     | VOLUME            |
|--|---------------------|-------------------|-------------------|
|  | Nuclease-free water | -                 | 80 $\mu\text{l}$  |
|  | BCXX-Rest-F1        | 100 $\mu\text{M}$ | 3.5 $\mu\text{l}$ |
|  | BCXX-Pol-F2         | 100 $\mu\text{M}$ | 6.5 $\mu\text{l}$ |
|  | BCXX-All-R          | 100 $\mu\text{M}$ | 10 $\mu\text{l}$  |
|  | TOTAL               |                   | 100 $\mu\text{l}$ |

#### Note

100  $\mu\text{l}$  of Primer Mix sufficient to perform 40 reactions at a 50- $\mu\text{L}$  RT-PCR reaction volume

- Once the concentrated stock is added, vortex the new working solution for at least 00:00:10
- To maintain optimal use of the primers, limit freeze/thaw cycles, as this can lead to degradation over time. To avoid repeated thawing and freezing, prepare small aliquots of working solutions and store at -20  $^{\circ}\text{C}$ . Thawed working primer solutions can be kept at 4  $^{\circ}\text{C}$  for weeks or months to use as needed.

6 Select one of the two one-step RT-PCR kit protocols below:

- SuperScript IV One-Step RT-PCR System (Invitrogen)
- LunaScript Multiplex One-Step RT-PCR Kit (NEB)

## STEP CASE

### SuperScript IV One-Step RT-PCR System (Invitrogen)

9 steps



- 7 Preheat the thermocycler to 55 °C , with the heated lid set to 105 °C
- 8 Keep all components, reaction mixes, and samples On ice
- 9 Prepare a 25 µL Master Mix (Volume 1) per reaction if visualization on TapeStation or no visualization is intended, and a 50 µL Master Mix (Volume 2) if gel visualization after PCR is planned for amplification product verification. Mix the following components:

| COMPONENT                               | VOLUME 1     | VOLUME 2     |
|-----------------------------------------|--------------|--------------|
| Nuclease-free water                     | 8.5 µl       | 17 µl        |
| 2X Platinum™ SuperFi™ RT-PCR Master Mix | 12.5 µl      | 25 µl        |
| Primer Mix (20 µM working solution)     | 1.25 µl      | 2.5 µl       |
| SuperScript™ IV RT Mix                  | 0.25 µl      | 0.5 µl       |
| RNA template                            | 2.5 µl       | 5 µl         |
| <b>TOTAL</b>                            | <b>25 µl</b> | <b>50 µl</b> |

Mix gently, briefly centrifuge, and ensure all the components are at the bottom of the amplification tube.

- 10 Place the reaction tubes in the pre-heated thermocycler and select the following RT-PCR cycling conditions:

| STEP                                 | TEMPERATURE | TIME    | CYCLE |
|--------------------------------------|-------------|---------|-------|
| Reverse transcription                | 55°C        | 2 min   | 1     |
|                                      | 42°C        | 90 min  |       |
| RT inactivation/initial denaturation | 98°C        | 2 min   | 1     |
| Amplification 1                      | 98°C        | 10 sec  | 5     |
|                                      | 45°C        | 30 sec  |       |
|                                      | 72°C        | 3.5 min |       |
| Amplification2                       | 98°C        | 10 sec  | 30    |
|                                      | 67°C        | 30 sec  |       |
|                                      | 72°C        | 3.5 min |       |



| STEP            | TEMPERATURE | TIME   | CYCLE |
|-----------------|-------------|--------|-------|
| Final extension | 72°C        | 10 min | 1     |
| Hold            | 4°C         | ∞      | -     |

#### Note

**Safe Stop Point:** If necessary, the protocol can be paused and samples can be kept at 4 °C overnight or at -20 °C for long-term storage, before proceeding to downstream applications.

A cleanup step is strongly recommended before downstream NGS library construction. We recommend using the **Select-a-Size DNA Clean & Concentrator** (Zymo Research, cat. number D4080), following the manufacturer's protocol to retain DNA fragments ≥ 300 bp.

## Quantification and Pooling

- 11 Quantify 1 µl of each sample using a Qubit fluorometer.
- 12 Adjust the input quantity of each sample prior to pooling, depending on the number of samples and the flow cell type. Calculate the required femtomoles (fmol) per sample by dividing the total recommended input by the number of samples to be pooled.

#### Note

Recommended input for the Ligation Sequencing Kit V14 (SQK-LSK114):

- **Flongle:** 50-100 fmol
- **MinION:** 100-200 fmol

- 13 Use the **NEBioCalculator** with the Qubit concentration values and an assumed average size of 2,000 bp for multi-segment RT-PCR fragments to calculate the molarity of each sample. Adjust the volume of each sample accordingly to meet the calculated fmol per sample required for the pool.
- 14 Pool samples based on these calculations to achieve the recommended input for sequencing.



- 15 Proceed to library preparation following the **Ligation Sequencing Amplicons V14 (SQK-LSK114)** protocol, starting from the DNA repair and end-prep step, followed by adapter ligation, according to the manufacturer's instructions.

## Protocol references

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