BPHL SARS-CoV-2 Tiled Amplicon Illumina Sequencing V.2

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This protocol details the Florida Department of Health's Bureau of Public Health Laboratories' (BPHL) wet lab portion of our SARS-CoV-2 next generation sequencing workflow. The method is a tiled amplicon approach using ARTIC V3 primers. The amplicon generation was adapted from the Matteson protocol[1]. The library preparation is Illumina NexteraXT. Library pooling and normalization were adapted from the Gohl protocol[3].

This protocol is for loading a MiSeq, but we have had equal success running on iSeqs and NextSeqs as well. Up to 96 libraries can be run on a MiSeq and up to 384 on a NextSeq.

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Cleaned up language in 4.10 for clarity/accuracy.

SARS-CoV-2, next generation sequencing, tiled amplicon

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RNA Extraction

1 Extract RNA from positive COVID-19 clinical specimens with the KingFisher Flex instrument using the Applied Biosystems™MagMAX™ Viral/Pathogen II (MVP II) Nucleic Acid Isolation Kit and its associated protocol.

cDNA Generation

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2 cDNA from RNA (from any extraction method) is produced using SuperScript™ IV VILO™ Master Mix **Thermo Fisher Scientific Catalog #11756500**
with the following ratios, per sample:

- **4 µL SuperScript IV VILO Master Mix**
- **6 µL Nuclease-free water**
- **10 µL Viral RNA**

2.1 Tightly seal reaction wells, mix reaction components with plate mixer and spin down

2.2 Run the following thermal cycler protocol:

- **25 °C 10 minutes**
- **50 °C 10 minutes**
- **85 °C 5 minutes**
- **4 °C ∞**

2.3 Store cDNA at -20ºC

ARTIC Amplicon Generation

3 ARTIC amplicons are produced by preparing two PCR reactions per sample (primer pool 1 is one reaction, primer pool 2 is the other). Per sample, combine below reagents in the listed ratios:

- **12.5 µL Q5 HI-FIDEL 2X MASTER MIX 500 rxn**
- **1 µL IDT ARTIC V3 Primer Pool (20uM)**
- **9 µL nuclease-free water**
- **2.5 µL cDNA**

3.1 Tightly seal reaction wells, mix reaction components with plate mixer and spin down

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3.2 Run the following thermal cycler protocol:

98 °C 30 seconds

95 °C 15 seconds

64 °C 5 minutes

total of 35 cycles of steps 2 and 3

4 °C ∞

3.3 Combine PCR-amplified DNA from primer pool 1 and 2 together and dilute to 0.2-0.6 ng/µL (for Illumina)

3.4 Proceed to Illumina library prep method of choice (NexteraXT, Flex, Illumina compatible)

Library Quantification & Pooling

4 Quantify the DNA concentration of each clean library using the Qubit High Sensitivity dsDNA kit

4.1 Pool equal concentrations (e.g., 1-10 ng) of each library. Total volume does not matter

4.2 Concentrate using 0.7x AMPureXP beads (ex. for 240 µL add 168 µL of beads)

4.3 Allow binding at room temperature for at least 5 minutes before clearing with magnet

4.4 Wash beads 2x with 80% EtOH while still on magnet

4.5 Remove all EtOH and allow to pellet to dry for 5 minutes

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4.6 Remove tube from magnet and add 20 µL Tris-HCl pH 8.0 to pellet. Slowly pipette mix

4.7 Incubate at room temperature for at least 5 minutes before clearing with magnet

4.8 Check DNA fragment distributions of the pooled sample. Peak fragment size from 400 bp tiled amplicons with proper ligated adaptors should be ~ 500nt

4.9 Quantify the DNA concentration of the pooled library using the Qubit High Sensitivity DNA kit

Note: At least 0.76 ng/µL is required to achieve 2 nM for library pooling. Libraries will need to be concentrated or re-amplified if less than this amount.

4.10 Convert DNA libraries from ng/uL to moles:
concentration [nM] = concentration [ng/µL] / ((ave.library size x 660)/1,000,000)

Example: if average size of library is 580 bp and concentration is 2.5 ng/µL:
(580 x 660) / 1,000,000 = 0.382
2.5 / 0.382 = 6.5 nM

4.11 Dilute the pooled library to 2 nM in 10 mM Tris pH 8.0

Final Dilution and Loading

5 Add 10 µL of a 2 nM library to 10 µL of freshly prepared 0.2 N NaOH

5.1 Mix by flicking, spin down, incubate at room temperature for 5 minutes

5.2 Add 980 µl prechilled HT1to the tube containing denatured library. The result is 1 ml of a 20 pM denatured library

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5.3 Add 240 µL of the 20 pM denatured library, 348 µl prechilled HT1, and 12 µL of 20 pM denatured PhiX. Invert to mix, spin down

5.4 The result is 600 µL of an 8 pM denatured library with 5% PhiX ready to be loaded on a MiSeq v3 cartridge

References

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