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SARS-CoV-2 Amplicon-based Illumina Sequencing Protocol

wgscov [1]

[1]Public Health Ontario

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ARTIC amplicon sequencing protocol adapted from Josh Quick's https://www.protocols.io/view/ncov-2019-sequencing-protocol-v2-bdp7i5rn for Illumina sequencing of SARS-CoV-2

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Preamble to cDNA Synthesis

1 Use *either* a SuperScript (step 2) or LunaScript kit (step 3) for cDNA synthesis

cDNA Synthesis 1h 30m

2 **Option 1: SuperScipt** mastermix

2.1 In a clean room, mix 1:1 dNTP and random hexamers. Aliquot **2 µL** per reaction, and add **11 µL** RNA, as per the table below. Seal plate and gently mix and centrifuge briefly to collect liquid at the bottom of the well.

Component	Volume
50 µM random hexamers	**1 µL**
10mM dNTPs mix (10mM each)	**1 µL**
Total Mastermix volume	**2 µL**
(template RNA)	**11 µL**
Total Reaction volume	**13 µL**

The Mastermix should be prepared in a clean room and added to the nucleic acid in a BSC exclusive for RNA work.

2.2 Incubate the reaction in a thermocycler as follows: 5m

Step	Temperature	Time	Cycles
1	**65 °C**	**00:05:00**	1
2	**4 °C**	Hold	

2.3 Prepare the following SuperScipt mastermix:

Component	Volume
SSIV Buffer	**4 µL**
100mM DTT	**1 µL**
RNaseOUT RNase Inhibitor	**1 µL**
SSIV Reverse Transcriptase	**1 µL**
Total Mastermix volume	**7 µL**
(denatured RNA)	**13 µL**
Total Reaction volume	**20 µL**

Add **7 µL** of mastermix to the denatured RNA from the previous step. Cover the plate with seal, mix gently on a plate mixer, and pulse spin the plate to collect liquid at the bottom of the tube.

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The Mastermix should be prepared in in a clean room and added to the denatured RNA in a BSC or workbench exclusive for RNA work.

2.4 Incubate in a thermocycler as follows: 1h

Step	Temperature	Time	Cycles
1	42 °C	00:50:00	1
2	70 °C	00:10:00	1
3	4 °C	Hold	

3 **Option 2: LunaScript** mastermix

3.1 In a clean room, mix the components as per the table below:

Component	Volume
Nuclease-free water	5 µL
LunaScript RT SuperMix (5x)	4 µL
Total Mastermix volume	9 µL
(template RNA)	11 µL
Total Reaction volume	20 µL

The Mastermix should be prepared in a clean room and added to the nucleic acid in a BSC exclusive for RNA work.

3.2 Incubate the reaction in a thermocycler as follows: 25m

Step	Temperature	Time	Cycles
1	25 °C	00:02:00	1
2	55 °C	00:20:00	1
3	95 °C	00:01:00	1
4	4 °C	00:02:00	1

Multiplex PCR Amplification 5m 45s

4 Prepare two multiplex PCR mastermixes as follows (1 for each pool):

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Component	Pool 1	Pool 2
5X Q5 Reaction Buffer	5 µL	5 µL
10 mM dNTPs	0.5 µL	0.5 µL
Q5 Hot Start DNA Polymerase	0.25 µL	0.25 µL
ARTIC Primer Pool 1 or 2 (10µM)	3.6 µL	3.6 µL
Nuclease-free water	13.15 µL	13.15 µL
Total Mastermix volume	22.5 µL	22.5 µL
(cDNA)	2.5 µL	2.5 µL
Total reaction volume	25 µL	2.5 µL

Prealiquot 22.5 µL of each mastermix (pool1 and pool2) to each plate (pool1 and pool2) accordingly. Add 2.5 µL cDNA to each well of both plates. Seal the plate, mix gently and centrifuge briefly to collect liquid at the bottom of the well.

5 Run the 3.5 hours PCR program for each pool: 5m 45s

Step	Temperature	Time	Cycles
Heat Activation	98 °C	00:00:30	1
Denaturation	98 °C	00:00:15	35
Annealing	65 °C	00:05:00	35
Hold	4 °C		1

Amplicon Pooling and Cleanup 22m 30s

6 Pool 12.5 µL of each pool 1 and 2 together (total 25µl) in an 0.2 ml 96 well PCR plate.

Perform AMPure XP bead cleanup according to directions, as follows.

6.1 Add 25 µL of AMPure XP (well-vortexed, room temperature) to the sample plate. Cover the plate with seal, mix gently on a plate mixer, and pulse spin the plate to collect liquid at the bottom of the tube. Incubate at Room temperature for 00:05:00 . 5m

6.2 Place the plate on a magnetic rack for 00:05:00 , or until the beads have 5m

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pelleted and the supernatant is completely clear.

6.3 Remove and discard the liquid from each well with a multichannel pippette, being careful not to touch the bead pellet.

6.4 Add **200 µL** of freshly prepared, room temperature 80% ethanol to each well, incubate for **00:00:30**, remove the ethanol carefully with a multichannel pipette. 30s

6.5 Repeat ethanol wash (step 6.4).Discard all ethanol and carefully remove as much residual ethanol as possible using a multichannel pipette. With the plate uncovered, incubate for **00:03:00** to **00:05:00** or until the pellet loses its shine (if the pellet dries completely it will crack and become difficult to resuspend). 8m

6.6 Remove from magnetic rack, add **28 µL** of EB buffer to wells and mix gently on a plate mixer, ensuring beads are well re-suspended. Briefly centrifuge the plate to collect the liquid at the bottom of the wells. Incubate at room temperature for **00:05:00**. 5m

6.7 Place the plate on magnetic rack and incubate for **00:02:00** to **00:05:00** or until the beads have pelleted and the supernatant is completely clear. 7m

6.8 Transfer **25 µL** of the clear supernatant to a new plate, ensuring no beads are transferred.

Gel Electrophoresis

7 Optional section; use remaining volumes from Pool 1 and Pool 2 to confirm amplification by gel electrophoresis.

7.1 Prepare 1% agarose gels with enough wells to load all samples. Load **2 µL** of a 100 bp ladder into gel on either side of each row of wells.

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7.2 Dispense **2 µL** of 6X loading dye into each sample with a multichannel pipette, mix and load **2 µL** of this mix into the gel.

7.3 Run at 240V for **00:20:00** . Visualize PCR products, confirm bands of approximately 300bp size. 20m

Amplicon Quantification and Normalization

8 Quantify amplicons using Qubit dsDNA High Sensitivity kit and plate reader according to directions, as follows.

8.1 Create Qubit dsDNA HS working solution by mixing **99.5 µL** X buffer and **0.5 µL** X dye (X is the total number of samples, including 6 standards). Using a reservoir and multichannel pipette, dispense **98 µL** into required number of wells of a Costar 3590 flat-bottom plate (or as appropriate for plate reader).

8.2 Dilute the clean, pooled amplicons (from step 6.8) 1:10 by mixing **3 µL** of the amplicons in **27 µL** of nuclease free water.

8.3 Make up serial standards using 1:2 dilutions of 10 ng/ul stock (Standard 2) from the Qubit HS. This creates 5 standards in the following concentrations: [M]**10 ng/ul** [M]**5 ng/ul** [M]**2.5 ng/ul** [M]**1.25 ng/ul** [M]**0.625 ng/ul** plus Standard 1 [M]**0 ng/ul** .

8.4 Mix **2 µL** of diluted amplicons and each of the 6 standards, **98 µL** of Qubit HS working solution, mix and breifly centrifuge. Use plate reader to obtain concentration reading for each sample and standards. The Qubit standard curve is generated by the Qubit standards.

9 Based on the amplicon concentration, normalize of all the samples amplicon concentration to [M]**0.2 ng/ul** .

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This can be done by adding **2.5 µL** of diluted amplicon to a plate with prealiquoted, appropriate amount of nuclease free water.

Library Preparation

10 Prepare sequencing libraries with Nextera XT DNA Library Prep kit at half volume, as follows.

11 **Tagment DNA.**

Thaw the following Nextera XT reagents on ice:

Amplicon tagment mix (ATM)
Tagment DNA buffer (TD)
Nextera PCR master mix (NPM)

Invert all reagents 3 - 5 times, followed by pulse spin.

11.1 1m
Add the following reagents in order:
5 µL of TD buffer
2.5 µL of 0.2 ng/ul amplicon (from step 9)
2.5 µL of ATM

Cover plate with plate seal, mix gently on plate mixer and centrifuge for **00:01:00** .

11.2 5m
Incubate in thermocycler with the following steps:

Step	Temperature	Time	Cycles
1	**55 °C**	**00:05:00**	1
2	**10 °C**	Hold	

11.3 5m
Remove the plate immediately once thermocycler reachs **10 °C** , and proceed to neutralization.
Add **2.5 µL** of NT buffer to each well and mix by pipetting up and down for 3 times, briefly spin down the plate and incubate at room temperature for **00:05:00** .

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12 PCR Amplification.

Thaw the following reagents on ice:
NPM
Index primers
Resuspension buffer (RSB)

Thaw the index primers, mix by vortexing each vial and spin down the liquid at the bottom of the vials. Option to dispense indexes into 96 well plate for easier pipetting.

12.1 Add **7.5 µL** of Nextera PCR mastermix to each well.

12.2 From the pre-aliquoted index plate, add **5 µL** (**2.5 µL** of each i5 and i7 index) of the corresponding index combination to each well. Cover plate with plate seal, gently mix on plate mixer, and centrifuge for **00:01:00** . 1m

12.3 Run the PCR program to amplify the libraries: 9m 40s

Step	Temperature		Time	Cycles
1	**72 °C**	**00:03:00**	1	
2	**95 °C**	**00:00:30**	1	
3	**95 °C**	**00:00:10**	12	
3	**55 °C**	**00:00:30**	12	
3	**72 °C**	**00:00:30**	12	
4	**72 °C**	**00:05:00**	1	
5	**4 °C**	Hold	1	

Library Cleanup

13 Repeat the same clean up process as step 6.1-6.8 using **20 µL** of AMPure XP beads and **28 µL** of resuspension buffer.

Library Quantification

14 Repeat the same quantification process as Step 8 but do NOT dilute libraries.

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Normalization and Loading on Illumina Sequencing Instrument 11m

15 Normalize each library to [M]**4 nanomolar (nM)** by dilution with nuclease free water.

16 Pool equal volume (e.g. **5 µL**) from each of the normalized libraries into a single 1.5 mL microtube.

17 Verify fragment size and concentration using Agilent D5000 Assay on TapeStation 4200 as follows.

17.1 Add **2 µL** of Sample Buffer and **2 µL** of your pooled libraries in triplicate in a strip tube.

17.2 Vortex using the adapter at 2000 rpm for **00:01:00** . 1m

17.3 Load tubes, tapes, and tips into TapeStation. Start run. Using library concentration and fragment size, calculate the molarity of the libraries using the following formula:
Molarity = concentration ng/uL * (1515.1515/fragment size(bp))

18 Denature and load pooled libraries for **MiSeq** as follows.

NOTE: Remove sequencing kit components from freezer to thaw at appropriate temperature/time.

18.1 Denature the pooled libraries by mixing **5 µL** of pooled libraries and **5 µL** of freshly made 0.2N NaOH solution.
Incubate for **00:05:00** . 5m

18.2 Add **990 µL** of HT1 buffer and mix well with denatured pooled library by pipetting up and down 10 times with P1000.

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18.3 Load **600 µL** of the denatured, diluted pooled library into the loading position of the Illumina MiSeq reagent cartridge (V2, 300 cycle kit). Load reagent cartridge, flow cell, and PR2 buffer into Miseq instrument, confirm the metrics and start the run.

19 For NextSeq loading, combine up to four pools of libraries at equal concentrations. Be careful to use unique index combinations for all pooled samples.

Denature and load pooled libraries for **NextSeq** as follows.

NOTE: Remove sequencing kit components from freezer to thaw at appropriate temperature/time.

19.1 Denature the pooled libraries by mixing **5 µL** of pooled libraries and **5 µL** of freshly made 0.2N NaOH solution. 5m

Incubate for **00:05:00** .

19.2 Add **5 µL** 200 mM TrisHCl pH7.5 and **985 µL** HT1 buffer,

19.3 Pipette **97 µL** of this denatured library solution into a new tube and add **1203 µL** of HT1 buffer.

19.4 Load **1300 µL** of the denatured, diluted pooled library into the loading position of the Illumina NextSeq reagent cartridge. Follow the prompts in the instrument to complete loading of flow cell, buffer bottle or cartridge and reagents cartridge

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