SARS-CoV-2 Illumina MiSeq protocol v.2 V.2

Public Health Ontario¹

¹Public Health Ontario Laboratory

wgscov

DISCLAIMER

DISCLAIMER – FOR INFORMATIONAL PURPOSES ONLY; USE AT YOUR OWN RISK

The protocol content here is for informational purposes only and does not constitute legal, medical, clinical, or safety advice, or otherwise; content added to protocols.io is not peer reviewed and may not have undergone a formal approval of any kind. Information presented in this protocol should not substitute for independent professional judgment, advice, diagnosis, or treatment. Any action you take or refrain from taking using or relying upon the information presented here is strictly at your own risk. You agree that neither the Company nor any of the authors, contributors, administrators, or anyone else associated with protocols.io, can be held responsible for your use of the information contained in or linked to this protocol or any of our Sites/Apps and Services.

ABSTRACT

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

License: This is an open access protocol distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

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

Created: Mar 12, 2021
cDNA preparation

1. Mix the following components:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 µM random hexamers</td>
<td>1 µL</td>
</tr>
<tr>
<td>10mM dNTPs mix (10mM each)</td>
<td>1 µL</td>
</tr>
<tr>
<td><strong>Total Mastermix volume</strong></td>
<td>2 µL</td>
</tr>
<tr>
<td>(template RNA)</td>
<td>11 µL</td>
</tr>
<tr>
<td><strong>Total Reaction volume</strong></td>
<td>13 µL</td>
</tr>
</tbody>
</table>

Prepare Mastermix (1:1) of random hexamers and dNTP.

Mix gently and pulse centrifuge to collect liquid at the bottom of the Mastermix tube.

**Note**

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

2. Aliquot 2 µL of this mix into each well of a 96 well plate. Keep the plate in a cold block.

3. Use multichannel pipette to aliquot 11 µL of RNA to the plate from step 2. Seal plate, mix gently on plate mixer, and briefly centrifuge the plate to collect the liquid at bottom of the wells.

4. Incubate the reaction mix in thermocycler as follows:
Prepare the following mastermix:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSIV Buffer</td>
<td>4 µL</td>
</tr>
<tr>
<td>100 mM DTT</td>
<td>1 µL</td>
</tr>
<tr>
<td>RNaseOUT RNase Inhibitor</td>
<td>1 µL</td>
</tr>
<tr>
<td>SSIV Reverse Transcriptase</td>
<td>1 µL</td>
</tr>
<tr>
<td><strong>Total Mastermix volume</strong></td>
<td>7 µL</td>
</tr>
<tr>
<td>(denatured RNA)</td>
<td>13 µL</td>
</tr>
<tr>
<td><strong>Total Reaction volume</strong></td>
<td>20 µL</td>
</tr>
</tbody>
</table>

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.

**Note**

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.

Incubate in a thermocycler as follows:

- 42 °C for 00:50:00
- 70 °C for 00:10:00
- 4 °C Hold

Prepare the multiplex PCR reactions as follows and aliquot in each well of a 96-well plate x2 (1 for each pool):

- **Multiplex PCR**
- 4h

protocols.io |  
[https://dx.doi.org/10.17504/protocols.io.bs98nh9w](https://dx.doi.org/10.17504/protocols.io.bs98nh9w)  
Oct 4 2021
### Component Table

<table>
<thead>
<tr>
<th>Component</th>
<th>Pool 1</th>
<th>Pool 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X Q5 Reaction Buffer</td>
<td>5 µL</td>
<td>5 µL</td>
</tr>
<tr>
<td>10 mM dNTPs</td>
<td>0.5 µL</td>
<td>0.5 µL</td>
</tr>
<tr>
<td>Q5 Hot Start DNA Polymerase</td>
<td>0.25 µL</td>
<td>0.25 µL</td>
</tr>
<tr>
<td>Primer Pool 1 or 2 (10µM)</td>
<td>3.6 µL</td>
<td>3.6 µL</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>13.15 µL</td>
<td>13.15 µL</td>
</tr>
<tr>
<td>Total Mastermix volume (cDNA)</td>
<td>22.5 µL</td>
<td>22.5 µL</td>
</tr>
<tr>
<td>Total reaction volume</td>
<td>25 µL</td>
<td>25 µL</td>
</tr>
</tbody>
</table>

Prealiquot 22.5 µL of each mastermix (pool1 and pool2) to each plate (pool1 and pool2) accordingly.

8. In a BSC or workbench exclusive for RNA work, add 2.5 µL of cDNA from step 6 to each 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.

9. Run the 3.5 hours PCR program for each pool:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat Activation</td>
<td>98 °C</td>
<td>00:00:30</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>98 °C</td>
<td>00:00:15</td>
<td>35</td>
</tr>
<tr>
<td>Annealing</td>
<td>65 °C</td>
<td>00:05:00</td>
<td>35</td>
</tr>
<tr>
<td>Hold</td>
<td>4 °C</td>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>

#### Amplicon Clean-up

10. Combine the two pools of amplicons:

Add 12.5 µL of each Pool 1 and Pool 2 (total 25µl) in an 0.2 ml PCR plate (low binding plate).

11. Perform AMPure XP bead cleanup according to directions, as follows.
11.1 Add 25 µL of AMPure XP beads (well-vortexed and at Room temperature) to the combined amplicons 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.

11.2 Place the plate on a magnetic rack for 00:05:00, or until the beads have pelleted and the supernatant is completely clear.

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

11.4 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.

11.5 Repeat ethanol wash (step 11.3 and 11.4).

11.6 Discard all ethanol and carefully remove as much residual ethanol as possible using a multichannel pipette. With the plate uncovered, incubate for 3-5 min or until the pellet loses its shine (if the pellet dries completely it will crack and become difficult to resuspend).

11.7 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.

11.8 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.
11.9 Transfer 25 µL of the clear supernatant to a new plate, ensuring no beads are transferred.

**Gel electrophoresis**

12 Use remaining volumes from Pool 1 and Pool 2 to confirm amplification (step 9). Make 1% agarose gels with enough wells for all samples.

13 Load 2 µl of the 100 bp ladder into gel on either side of each row of wells.

14 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.

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

**Amplicon quantification and normalization**

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

16.1 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).
16.2 Dilute the clean, pooled amplicons (from step 11.9) 1:10 by mixing 3 µL of the amplicons in 27 µL of nuclease free water.

16.3 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

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

17 Based on the amplicon concentration, normalize of all the samples amplicon concentration to 0.2 ng/ul. This can be done by adding 2.5 µL of diluted amplicon to a plate with pre aliquoted, appropriate amount of nuclease free water.

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

19 Tagment DNA. 

Thaw the following Nextera XT reagents on ice:
- Amplicon tagment mix (ATM)
- Tagment DNA buffer (TD)
- Nextera PCR master mix (NPM)
- Thaw the index primers, mix by vortex each vial and spin down the liquid at the bottom of the vials.
- Neutralization buffer (NT) at Room temperature

19.1 Add the following reagents in order:
1. 5 µL of TD buffer
2. 2.5 µL of [M] 0.2 ng/ul amplicon (from step 17)
3. 2.5 µL of ATM

Cover plate with plate seal, mix gently on plate mixer and centrifuge for 1 min.

19.2 Incubate in thermocycler with the following steps:

- 55 °C 00:05:00
- 10 °C hold

19.3 Remove the plate immediately once thermocycler reaches 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.

20 PCR Amplification.

Thaw the following reagents on ice:
- NMP
- Index primers

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

20.1 Add 7.5 µl of Nextera PCR mastermix to each well.

20.2 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 1 min.

20.3 Run the PCR program to amplify the libraries:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>72 °C</td>
<td>00:03:00</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>95 °C</td>
<td>00:00:30</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>95 °C</td>
<td>00:00:10</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>55 °C</td>
<td>00:00:30</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>72 °C</td>
<td>00:00:30</td>
<td>12</td>
</tr>
<tr>
<td>4</td>
<td>72 °C</td>
<td>00:05:00</td>
<td>1</td>
</tr>
</tbody>
</table>
Clean Up Libraries

Repeat the same clean up process as step 11 using 20 µL of AMPure XP beads and 28 µL of resuspension buffer.

Library Quantification

Repeat the same quantification process as Step 16 but do NOT dilute libraries.

Normalization and loading on Miseq

Normalize each library to 4 nanomolar (nM) by dilution with nuclease free water.

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

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

Add 2 µl of Sample Buffer and 2 µl of your pooled libraries in triplicate in a strip tube.

Vortex using the adapter at 2000 rpm for 1 min.
25.3 Load tubes, tapes, and tips. Start run. Using library concentration and fragment size, calculate the molarity of the libraries using the following formula:
Molarity = concentration ng/µL * (1515.1515/fragment size(bp))

26 Denature and load pooled libraries as follows.

26.1 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.

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

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