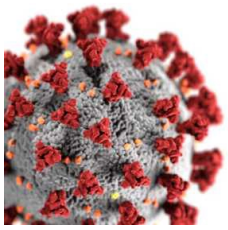
Oct 04, 2021 Version 2

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



DOI
dx.doi.org/10.17504/protocols.io.bs98nh9w

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DOI: dx.doi.org/10.17504/protocols.io.bs98nh9w

Protocol Citation: Public Health Ontario 2021. SARS-CoV-2 Illumina MiSeq protocol v.2. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bs98nh9wVersion created by **wgscov**

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

Created: March 12, 2021

Last Modified: October 04, 2021

Protocol Integer ID: 48160

Disclaimer

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

cDNA preparation

10m

1 Mix the following components: 15m

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

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. 5m

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. 5m

4 Incubate the reaction mix in thermocycler as follows: 6m

65 °C 00:05:00
4 °C 00:01:00

5 Prepare the following mastermix: 15m

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.

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.

6 Incubate in a thermocycler as follows: 1h

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

Multiplex PCR 4h

7 Prepare the multiplex PCR reactions as follows and aliquot in each well of a 96-well plate x2 (1 for each pool): 10m

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
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	25 µL

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. 10m

9 Run the 3.5 hours PCR program for each pool: 3h 30m

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 Clean-up 1h

10 Combine the two pools of amplicons: 5m
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. 45m

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 pippette, 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

1h

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. 20m

13 Load 2 µl of the 100 bp ladder into gel on either side of each row of wells. 5m

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. 20m

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

Amplicon quantification and normalization 2h

16 Quantify amplicons using Qubit dsDNA High Sensitivity kit and plate reader according to directions, as follows. 30m

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 plus Standard 1 [M] 0 ng/ul standard 1 .

16.4 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.

17 Based on the amplicon concentration, normalize of all the samples amplicon concentration to [M] 0.2 ng/ul .
This can be done by adding 2.5 µL of diluted amplicon to a plate with prealiquoted, appropriate amount of nuclease free water. 30m

Library preparation 2h

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: 30m

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 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 .

20 PCR Amplification. 1h
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:

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 Clean-up

2h

21 Clean Up Libraries

45m

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

Library Quantification

2h

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

30m

Normalization and loading on Miseq

2h

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

30m

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

15m

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

20m

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

25.2 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/uL * (1515.1515/fragment size(bp))

26 Denature and load pooled libraries as follows. 10m

26.1 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 .

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.