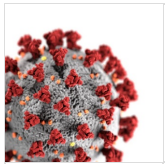

Aug 23, 2020

# SARS-CoV-2 Illumina MiSeq protocol v.1

Public Health Ontario[1]

[1]Public Health Ontario Laboratory

1 Works for me This protocol is published without a DOI.

Coronavirus Method Development Community

wgscov

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

PROTOCOL CITATION

Public Health Ontario 2020. SARS-CoV-2 Illumina MiSeq protocol v.1. **protocols.io** https://protocols.io/view/sars-cov-2-illumina-miseq-protocol-v-1-bjd9ki96



CREATED

Aug 06, 2020

LAST MODIFIED

Aug 24, 2020

PROTOCOL INTEGER ID

40097



## cDNA preparation 10m

1 Mix the following components:

| 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 by pipetting up and down and pulse centrifuge to collect liquid at the bottom of the mastermix tube.

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 pippet to aliquot **11 µl** of RNA to the plate from step 2, mix up and down 5x by multichannel pippette, briefly centrifuge the plate to collect the liquid at bottom of the wells.

4 Incubate the reaction mix in thermocycler as follows:

**65 °C** **00:05:00**

**4 °C** Hold

cDNA preparation 1h

5 Prepare the following 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. Mix gently by pipetting up and down and briefly centrifuge to collect liquid at the bottom of the well.

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 Perform reverse transcription reaction in thermocycler as follows:

| | |
|---|---|
| 42 °C | 00:50:00 |
| 70 °C | 00:10:00 |
| 5 °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):

| 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 and mix well by pipetting up and down. Seal the plates and briefly centrifuge to collect liquid at the bottom of the wells.

9 Run the 3.5 hours PCR program for each pool:

| 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 |

Gel Electrophoresis 1h

10 Prepare large 1% agarose gels with TBE buffer with enough wells for each PCR product and a ladder on each row.

11 Remove plates Pool 1 and Pool 2 amplicons from thermocyclers, briefly centrifuge.

12 Mix 2 µl of 6X loading dye with 2 µl of PCR product and load gels with Pool 1 amplicons leaving a space between each sample to be filled by Pool 2 amplicons so that each sample can be visualized for both pools. Add 100bp ladder to left- and right-most wells.

13 Run gels electrophoresis at **140V** for **00:20:00**

14 Confirm that presence/absence of bands in gels correlate RT-PCR Ct values for each sample (e.g. if band is absent, does sample have E-gene Ct >30?). Band sizes should be approximately 400bp.

## Amplicon Clean-up 1h

15 Combine the two pools of amplicons into one:
Add **12.5 µl** of each Pool 1 and Pool 2 (total 25µl) in an **0.2 ml PCR plate (low binding plate)**.

16 Add **25 µl** of AMPure XP beads(at **Room temperature** ) to the combined amplicons plate. Mix by pippetting up and down 10x, briefly centrifugre, and incubate for **00:05:00** .

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

16.2 Remove and discard the liquid from each well with a multichannel pippette.

16.3 Add **200 µl** of freshly prepared 80% ethanol to each well, incubate for **00:00:30** , remove the ethanol carefully with a multichannel pippette.

16.4 Repeat ethanol wash (Step 16.3).

16.5 Remove the residual ethanol with a P20 multichannel pippette, allow plate to dry for 3-5 minutes on the magnetic rack or until the pellet loses its shine (do not over-dry the pellet).

16.6 Remove from magnetic rack, add **28 µl** of EB buffer to wells and mix thoroughly by multichannel pippetting, until beads are resuspended. Briefly centrifuge the plate to collect the liquid at the bottom of the wells. Incubate at **Room temperature** for **00:05:00** .

16.7 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.

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

## Amplicon quantification and normalization 2h

17

Quantify amplicons using Qubit dsDNA High Sensitivity and DTX880 plate reader.

17.1 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.

17.2 Dilute the amplicon (from step 16.8) 1:10 by mixing **3 µl** of the amplicon in **27 µl** of nuclease free water.

17.3 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: **10 ng/ul** **5 ng/ul** **2.5 ng/ul** **1.25 ng/ul** **0.625 ng/ul** plus Standard 1 **0 ng/ul standard 1** .

17.4 Mix **2 µl** of diluted amplicons and each of the 6 standards **98 µl** of Qubit HS working solution, mix and breifly centrifuge. Use DTX880 plate reader to obtain reading for each sample and standards. The Qubit standard curve is generated by the Qubit standards.

18 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 prealiquoted and appropriate amount of nuclease free water.

Library preparation 2h

19 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 bottowm of the vials.
Neutralization buffer (NT) at **Room temperature**

20 Add the following reagents in order:
1. **5 µl** of TD buffer
2. **2.5 µl** of **0.2 ng/ul** amplicon
3. **5 µl** of ATM

Mix by pipetting up and down for 5 times.

21 Seal plate and briefly centrifuge. Incubate in thermocycler with the following steps:

**55 °C** **00:05:00**
**10 °C** hold

22 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** .

23 Place the i5 and i7 index primers on the Illumina Index Adapter plate accordingly,
Add **7.5 µl** of NPM to each wells and mix by pipetting up and down for 3 times.

Add **2.5 µl** each of i5 and i7 index primers to each samples accordingly and mix by pipetting up and down for 5 times.
Seal plate and briefly centrifuge.

24 Run the 1 hour 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** | 13 |
| 3 | **55 °C** | **00:00:30** | 13 |
| 3 | **72 °C** | **00:00:30** | 13 |
| 4 | **72 °C** | **00:05:00** | 1 |
| 5 | **4 °C** | Hold | 1 |

25 Briefly spin the plate to collect liquid at the bottom of the wells.
Repeat the same clean up process as step 16 using **20 µl** of AMPure XP beads and **28 µl** of resuspension buffer (step 16.8).

26 Repeat the same quantification process as Step 17 but do NOT dilute libraries.

## Normalization and loading on Miseq 2h

27 Verify fragment size using Agilent D5000 Assay on TapeStation 4200.
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))

28 Normalize all the libraries to **4 Nanomolar (nM)** by dilution with nuclease free water.

29 Pool **5 µl** from each of the normalized libraries into a signal **1.5 mL** microtube.
Measure the final pool concentration in duplicate using HS D1000 tape station.

30 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** .

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

Further dilute the pool library down to **4 Nanomolar (nM)** with HT1 buffer to prevent over clusering, if the HS D1000 tape station concentration of the library shows greater than 4nM.

32 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.