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SARS-CoV-2 Tailed Amplicon Illumina Sequencing V.1

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UNIVERSITY OF MINNESOTA
GENOMICS CENTER

Daryl Gohl[1]
[1]University of Minnesota

Coronavirus Method De...

Daryl Gohl
University of Minnesota

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

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Abstract

This protocol outlines how to process RNA for SARS-CoV-2 sequencing using tailed primers to generate tiled amplicons using the method described here: https://www.biorxiv.org/content/10.1101/2020.05.11.088724v1.

Best results are obtained for samples with N1 and N2 Ct values of <30 (based on the UMGC/MDL implementation of the CDC qRT-PCR diagnostic assay for SARS-CoV-2, see here: https://www.biorxiv.org/content/10.1101/2020.04.02.022186v1.full). For samples with N1 and N2 values between 30 and ~35, coverage and other sequencing metrics may be more variable and increased adapter dimer formation is expected.

Materials

1) Fully skirted 96-well plate. (BioRad)
2) Semi-skirted 96-well plate (Thermo Scientific)
3) Nuclease-free water. (Fisher Scientific)
4) Microseal F foil seals. (BioRad)
5) Microseal B PCR seals. (BioRad)
6) SuperScript IV VILO master mix (Thermo)
7) Q5 Hot Start High Fidelity DNA polymerase. (NEB)
8) 10 mM dNTPs (NEB)
9) nCov-2019 pool 1.1, 1.2, 2.1, 2.2 primers. (IDT) – see Appendix
10) Indexing primers. (IDT) – see Appendix
11) Rainin Liquidator 96 pipette with p20/p200 tips. (Rainin)
12) Rainin single/multichannel pipettes with p20/p200/p1000 tips. (Rainin)
13) White Matrix troughs. (Thermo Scientific)
14) SequalPrep Normalization Plate Kit, 96-well. (Thermo Scientific)
15) AMPure XP beads. (Beckman Coulter)
16) Combinatorial Dual Indexing Primers:
For 384 sample barcoding scheme, see “Indexingprimers.xlsx”, from:
https://protocolexchange.researchsquare.com/article/nprot-4831/v1
17) Unique Dual Indexing Primers:
Available from Illumina (Nextera Unique Dual Indexing Primers, catalog number: 20027213, 20027214, 20027215, 20027216.

Troubleshooting

Before start

Tailed primers should be pooled to generate 4 primer pools (1.1, 1.2, 2.1, 2.2) according to the pooling scheme described in **Supplemental Data File 2** here:
https://www.biorxiv.org/content/10.1101/2020.05.11.088724v1.supplementary-material.

Set up

10m

1 **Clean workspace and pipets by spraying with RNaseZAP or comparable product (such as RNase Away) and wiping down with KimWipes prior to beginning work.**

RNA samples should be stored at -80 °C and thawed on ice.

cDNA synthesis

2 Thaw RNA samples on ice then transfer 5 µL of sample into a 96-well Thermo PCR plate.

3 Set up the following reverse transcription reaction master mix (multiply below volumes by number of reactions plus desired overage):

11 µL nuclease free water
4 µL SuperScript IV VILO master mix

4 Transfer 15 µL of reverse transcription master mix to each sample containing well.

5 Seal plate with a “B” seal, mix well by vortexing using a plate vortexer at 1900 rpm for 00:00:10 s, and spin down briefly in a plate centrifuge (00:00:05 s at 2500 rpm .

6 Incubate in a thermocycler using the following conditions:

25 °C for 00:10:00
50 °C for 00:10:00
85 °C for 00:05:00

Enrichment PCR

7 Transfer 2.5 µL of cDNA to each of 4 96-well Thermo PCR plates labeled: Project_Name_PCR1_1.1, Project_Name_PCR1_1.2, Project_Name_PCR1_2.1, and Project_Name_PCR1_2.2.

8 Set up the following four PCR master mixes, one for each of the four multiplexed primer pools (multiply below volumes by number of reactions plus desired overage):

14.75 µL nuclease-free water
5 µL 5x Q5 reaction buffer
0.5 µL 10mM dNTPs
0.25 µL Q5 Polymerase
2 µL primer pool (10 µM) (Either pool 1.1, 1.2, 2.1, or 2.2)

9 Transfer 22.5 µL of master mix to each well of the appropriate PCR plate.

10 Seal plate with a “B” seal, mix well by vortexing using a plate vortexer at 1900 rpm for 00:00:10, and spin down briefly in a plate centrifuge (00:00:05 at 2500 rpm).

11 Amplify samples using the following PCR conditions:

98 °C for 00:00:30

35 cycles of:
98 °C for 00:00:15
65 °C for 00:05:00

Indexing PCR

12 For each sample, combine 10 µL of each of the four pools in a single Bio-Rad fully-skirted 96 well plate.

13 Seal plate with a “F” seal, mix well by vortexing using a plate vortexer at 1900 rpm for 00:00:10, and spin down in a plate centrifuge (00:00:30 at 2500 rpm).

14 In a 96-well Thermo plate, add 2 µL of each sample to 198 µL of nuclease free water (1:100 dilution).

15 Seal plate with a “F” seal, mix well by vortexing using a plate vortexer at 2500 rpm for 00:00:10, and spin down in a plate centrifuge (00:00:30 at 2500 rpm).

16 Transfer 5 µL of 1:100 diluted PCR 1 sample to a 96-well Thermo PCR plate.

17 Transfer 2 µL of 5 µM indexing primer mix to the 96-well Thermo PCR plate containing the samples.

18 Set up the following PCR master mix (multiply below volumes by number of reactions plus desired overage):

- 0.7 µL nuclease-free water
- 2 µL 5x Q5 reaction buffer
- 0.2 µL 10 mM dNTPs
- 0.1 µL Q5 Polymerase

19 Transfer 3 µL of master mix to each well of the appropriate PCR plate.

20 Seal plate with a “B” seal, mix well by vortexing using a plate vortexer at 1900 rpm for 00:00:10, and spin down briefly in a plate centrifuge (00:00:05 at 2500 rpm).

21 Amplify samples using the following PCR conditions:

98 °C for 00:00:30

35 cycles of:
98 °C for 00:00:20
55 °C for 00:00:15

72 °C for 00:01:00

72 °C for 00:05:00

Normalization

22 Normalize samples using a SequalPrep plate according to manufacturer's instructions.
sequalprep_platekit_man.pdf

23 Elute in 20 µL of SequalPrep Elution Buffer.

Pooling

24 Pool 10 µL of each sample in a trough, mix well and transfer material to a 1.5 mL non-stick tube.

25 Purify using AMPureXP beads at a 0.7x ratio. Elute library in 20 µL of EB.

Library QC

26 Perform final QC on pool by determining concentration (PicoGreen or Qubit assay). Prepare 2 nM pool dilution, based on the sample concentration as determined by PicoGreen and fragment size (expected size is ~555 bp).

Sequencing

27 Dilute pooled sample to 8 pM in HT1, following MiSeq loading instructions, spike in 5% 8 pM PhiX, and load in MiSeq 2×250 or 2×300 reagent cartridge.