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SARS-CoV-2 Tailed Amplicon Illumina Sequencing

Daryl Gohl[1]

[1]University of Minnesota

1 Works for me dx.doi.org/10.17504/protocols.io.bge5jtg6

Daryl Gohl

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 TEXT

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.

BEFORE STARTING

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.

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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:

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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).

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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 2x250 or 2x300 reagent cartridge.

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