Demonstrated protocol for sequencing SARS-CoV-2 with the Midnight Panel and the Lotus DNA Library Prep Kit

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The method presented here is provided by IDT. This method can be used as a starting point for using SARS-CoV-2-Midnight-1200, 500 rxn, and the Lotus DNA Library Prep Kit in similar experiments but may not be fully optimized for your application. IDT does not guarantee this method, as it has not been fully verified and validated by our internal team. As a result, application scientists at IDT can only provide general guidance with limited troubleshooting support.

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OVERVIEW

COVID-19, caused by the coronavirus SARS-CoV-2, is an unprecedented global health challenge. The coronavirus has spread rapidly and causes a range of symptoms from mild upper respiratory effects to critical respiratory illness and even death. Understanding the biology of the virus is vital to combat it and reduce the clinical burden caused by this infection. Coronaviruses are RNA viruses and copy their genomic content directly, RNA to RNA. This is a relatively error-prone process, and over time mutations occur within the viral genome. Some of the mutations are neutral and randomly emerge and then disappear. Others provide some sort of advantage to the virus and become more common within the population. The emergence of new variants adds further challenges, leading to a need to track the virus.

Sequencing viral samples has been made more straightforward by amplification enrichment approaches that take total RNA from samples that include virus and human cells. Samples are reverse transcribed to form cDNA, and then specific amplification of the viral content is performed. To ensure complete viral genome coverage, two pools of primers are often used to separate overlapping amplicons. After amplification, the DNA is pooled and ligated to form an NGS-compatible library. Two sequencing platforms, Oxford Nanopore and Illumina, are in common use for this process.

The Midnight Panel was created by Drs. Nikki Freed and Olin Silander of Massey University in New Zealand [1]. This panel consists of 29 amplicons of approximately 1200 bp in length. The reduced number of amplicons allows for more even sequencing across the genome than would be achievable with a higher number of amplicons. The panel was designed for use on the Oxford Nanopore sequencing platform. The length of the amplicons presents a problem for use on Illumina sequencers, which do not have read length options to cover the entire amplicon. This means that fragmentation of the amplicons is required before library construction. Here we present the use of the Lotus DNA Library Prep Kit as a simple workflow (Figure 1) to solve this challenge. The protocol includes a simple enzymatic fragmentation step which quickly creates lengths compatible with Illumina sequencing. Data indicate this workflow is highly efficient and rapid.

1	cDNA synthesis	30 minutes
2	Amplicon generation	2.5–3.5 hours
3	Post-amplification cleanup	0.65X Ampure • 20 minutes
4	Fragmentation/End-repair/A-tail	Based on 200 bp fragment time in CoA
5	Ligation	30 minutes
6	Post-ligation cleanup	0.8X Ampure • 20 minutes
7	PCR amplification	20–45 minutes
8	Post-PCR cleanup	1.8X Ampure • 20 minutes
9	Purification	1.2X Ampure • 20 minutes

Figure 1. Workflow of the demonstrated protocol.

CONSUMABLES AND EQUIPMENT

Consumables from IDT

Item	Catalog #/URL
Lotus DNA Library Prep Kit	www.idtdna.com/Lotus 10001074 (96 rxn)
xGen™ Stubby Adapter and UDI Primer Pairs	www.idtdna.com/stubby-UDI 10005921 (96 rxn)
Nuclease-Free Water	11-04-02-01
SARS-CoV-2-Midnight-1200, 500 rxn	1007184
1 Liter IDTE, pH 8.0 (1X TE Solution)	11-05-01-09
IDT Duplex Buffer	11-01-03-01

Consumables from other suppliers

Item	Supplier	Catalog #
LunaScript™ RT SuperMix Kit	New England BioLabs	E3010L (100 rxn)
Q5® High-Fidelity 2X Master Mix	New England BioLabs	M0492S (100 rxn)
Absolute ethanol (200 proof)	General laboratory supplier	Varies
Agencourt® AMPure® XP–PCR purification beads, 5 mL	Beckman Coulter	A63880
twin.tec® PCR plate 96 LoBind®, semi-skirted	Eppendorf	0030129504
Library Quantification Kit–Illumina/Universal	Kapa Biosystems	KK4824
MAXYMum Recovery® Microtubes, 1.7 mL	Corning	MCT-175-L-C
Microseal® 'B' PCR Plate Sealing Film	Bio-Rad	MSB1001
Qubit™ dsDNA HS Assay Kit, or equivalent	Thermo Fisher Scientific	Q32851 or Q32854
High Sensitivity DNA Kit, or equivalent	Agilent	5067-4626
High Sensitivity D1000 ScreenTape™, or equivalent	Agilent	5067-5584

Equipment

Item	Supplier	Catalog #
2200 TapeStation™ System/4200 TapeStation System, or equivalent	Agilent	G2965AA or G2991AA
Qubit 4 Fluorometer, or equivalent	Thermo Fisher Scientific	Q33226
Microcentrifuge	General laboratory supplier	Varies
Plate centrifuge	General laboratory supplier	Varies
Thermal cycler	General laboratory supplier	Varies
qPCR system	General laboratory supplier	Varies
Magnet options (choose one):		
Magnum™ EX Universal Magnet Plate	Alpaqua	A000380
Magnetic Stand-96	Thermo Fisher	Q33226

TEMPLATE GENERATION [2]

cDNA synthesis

1. Thaw extracted RNA on ice.
2. Mix and briefly centrifuge all components prior to use.
3. Prepare the cDNA Master Mix as follows on ice:

Reagent	Volume per sample (µL)
LunaScript™ RT SuperMix	4
Nuclease-Free Water	6
Total volume	10

4. Mix thoroughly.
5. Add 10 µL of the cDNA Master Mix into a low-bind PCR plate containing 10 µL of extracted RNA.
6. Seal, briefly vortex, and centrifuge plate.
7. Place plate on a thermal cycler and run the following program:

Step	Temperature (°C)	Duration
Primer annealing	25	2 minutes
cDNA synthesis	55	20 minutes
Heat inactivation	95	1 minute
Hold	4	∞

Safe stopping point: Follow your established laboratory guidelines for proper storage of cDNA.

Amplicon generation [2]

Important! Primer pools 1 & 2 are intended to be used separately and should not be combined. Each primer pool will need to be diluted before use.

1. Prepare primer pools 1 & 2 by diluting the 100 µM Midnight primer stocks to 10 µM in IDTE.
2. Mix and briefly centrifuge all components prior to use.
3. Prepare Amplicon Master Mix as follows (one mix per primer pool):

Reagent	Volume per sample (µL)
Q5 Hot Start Master Mix	12.5
Midnight Primer Pool 1	1.1
Nuclease-Free Water	8.9
Total volume	22.5

Reagent	Volume per sample (µL)
Q5 Hot Start Master Mix	12.5
Midnight Primer Pool 2	1.1
Nuclease-Free Water	8.9
Total volume	22.5

4. Mix thoroughly.
5. Add 2.5 µL of cDNA to the appropriate wells of a low-bind PCR plate (See Note 1, Appendix A). Each sample will need two wells (one well per primer pool).
6. Add 22.5 µL of Amplicon Master Mix to the appropriate wells.
7. Seal, briefly vortex, and centrifuge plate.
8. Place plate on a thermal cycler, and run the following program:

Step	Temperature (°C)	Duration	Cycles
Heat Activation	98	30 seconds	1
Denaturation	98	15 seconds	25–35 (Cycle # may need optimization; see Note 2, Appendix A)
Annealing and Extension	65	5 minutes	
Hold	4	∞	1

Safe stopping point: Follow your established laboratory guidelines for proper storage of cDNA.

9. Allow AMPure XP beads to equilibrate to room temperature. Vortex until homogenous before use.
10. Briefly centrifuge amplified cDNA plate.
11. Combine the entire volume of primer pool 1 and primer pool 2 PCR reactions per sample (total volume of 50 µL amplified cDNA) in a new low-bind PCR plate.
12. Add 32.5 µL (0.65X) of AMPure XP beads per sample.

13. Mix well by pipetting.
14. Incubate at room temperature for 5 minutes.
15. Transfer sample plate to a magnet and allow beads to form pellet (~2 minutes or until clear).
16. Remove and discard the supernatant without disturbing the bead pellet.
17. Wash the beads with 180 µL of fresh 80% ethanol for 30 seconds, then remove the ethanol and discard. Do not disturb the pellet.
18. Repeat previous wash step for a total of two washes.
19. Using a new p20 pipette tip, remove any residual ethanol solution from the bottom of the well.
20. Allow beads to dry on the magnetic plate for 1–3 minutes.

    Note: Avoid over-drying beads, as this will reduce yield.

21. Remove sample plate from magnet, add 22 µL of Nuclease-Free Water, and resuspend by mixing.
22. Incubate at room temperature for 3 minutes.
23. Transfer sample plate back to the magnet, allow beads to collect for 5 minutes.
24. Carefully transfer 20 µL of supernatant to a new low-bind PCR plate, being careful to not disturb the pellet.

    Safe stopping point: Follow your established laboratory guidelines for proper storage of cDNA.

    Measure the concentration of the amplicon pool using a Qubit fluorometer (or equivalent) to ensure an input of 1–250 ng into the library preparation.

LIBRARY PREPARATION

Prepare reagents

1. Briefly vortex Lotus Ligation Buffer and keep at room temperature.
2. Place all kit enzymes on ice (not in a cryocooler) for at least 20 minutes to allow enzymes to reach 4°C before pipetting.
3. Thaw other kit reagents, buffers, and primers on ice (4°C). Briefly vortex to mix well.
4. Spin all tubes in a microcentrifuge to collect contents before opening.
5. Prepare a fresh 80% ethanol solution using absolute ethanol (200 proof) and Nuclease-Free Water. At least 1.5 mL of 80% ethanol solution will be used per sample.

Perform enzymatic preparation

Important! Keep the Enzymatic Prep Master Mix and the DNA samples on ice until they are loaded in the thermal cycler to safeguard against fragmentation; enzymes are active at room temperature and may fragment DNA to undesired sizes.

1. Transfer the amplified cDNA sample (1–250 ng) to a new low-bind PCR plate, adjust the volume to a total of 19.5 µL (if necessary) using Lotus Elution Buffer, and place sample plate on ice.

   Note: If not normalizing amplified cDNA concentration prior to library preparation, we recommend using 25 ng or more of input into the fragmentation.

Reagent	Volume per sample (µL)
Lotus Elution Buffer	(19.5 – x)
Amplified cDNA	x
Total volume	19.5

2. Set up the thermal cycler with the Enzymatic Prep program as described, with the heated lid set to 70°C:

Step	Temperature (°C)	Duration
Hold	4	∞
Enzymatic prep	32	See CoA
	65	30 minutes
Hold	4	∞

Note: See your Certificate of Analysis (CoA) for fragmentation time recommendations for the lot number you receive. Reaction times may need to be optimized for individual samples. Specifically, for sample inputs <25 ng, longer fragmentation may be required. To find the CoA, enter the lot number, which can be found on a label on the bottom of the kit, at www.idtdna.com/COA. We suggest using the 200 bp fragmentation time from the CoA.

The TapeStation trace (Figure 2) shows amplicon fragmentation using 16, 18, and 20 minute fragmentation times; the CoA recommendation for fragmentation to 200 bp using this lot of fragmentase was 16 minutes.

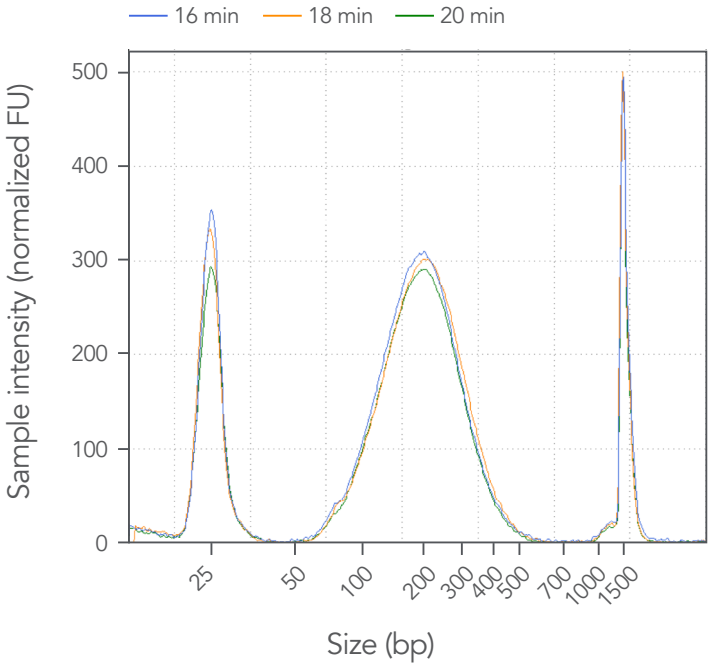

Figure 2. TapeStation trace showing effects of fragmentation times.

3. Begin Enzymatic Prep program to chill thermal cycler to 4°C.
4. Prepare the Enzymatic Prep Master Mix by adding the components in the order shown:

Reagent	Volume per sample (µL)
Lotus Enzymatic Prep Buffer	3
Lotus Enzymatic Prep Reagent*	1.5
Lotus Enzymatic Prep Enzyme	6
Total volume: Enzymatic Prep Master Mix	**10.5**

* If samples are in ≥1 mM EDTA, using 2–3X volume of Lotus Enzymatic Prep Reagent will reduce EDTA-induced under-fragmentation.

Note: Keep reagents and master mix on ice as much as possible.

5. Gently vortex the Enzymatic Prep Master Mix for 5 seconds.

Important! Thoroughly mix the Enzymatic Prep Master Mix before and after adding to your DNA samples. Because this master mix is viscous, failure to mix thoroughly could result in incomplete fragmentation.

6. Add 10.5 µL of premixed Enzymatic Prep Master Mix to each well containing DNA samples and Lotus Elution Buffer to reach a final volume of 30 µL.
7. Thoroughly vortex to mix for 5 seconds.
8. Briefly centrifuge the sample plate, then immediately place in the chilled thermal cycler, and advance the Enzymatic Prep program to the 32°C fragmentation step.
9. Continue the Enzymatic Prep program to completion.

Important! Fragmented samples can be kept at 4°C no more than 1 hour.

Perform Ligation

1. If you are using DNA inputs <25 ng, dilute the xGen Stubby Adapter in Duplex Buffer (IDT), as described:

DNA input (ng)	Adapter dilution	Stock concentration (µM)
≥25	No dilution	15
10	10-fold (1:10)	1.5
1	20-fold (1:20)	0.75

2. Prepare the Ligation Master Mix, adding the components in the order shown:

Reagent	Volume per sample (µL)
Lotus Elution Buffer	10
Lotus Ligation Buffer (room temperature)	12
Lotus Ligation Enzyme	4
Total volume: Ligation Master Mix	26

Important! Slowly pipette the viscous Lotus Ligation Buffer to avoid bubbles and ensure accuracy. Keep reagents and master mix on ice except for the Lotus Ligation buffer.

3. Gently vortex Ligation Master Mix for 5 seconds.
4. When the Enzymatic Prep program is complete, add 26 µL of premixed Ligation Master Mix to the plate containing your fragmented amplified cDNA samples (total volume is 56 µL).
5. Add 4 µL of adapter to the mixture and place sample plate on ice (total volume is 60 µL).

Note: For amplified cDNA inputs <25 ng, use diluted adapters from step 1 (above).

6. Thoroughly vortex to mix for 5 seconds.
7. Set up the thermal cycler with the Ligation program as described, with lid heating **OFF**, or s**et at 40°C**.

Step	Temperature (°C)	Duration (minutes)
Ligation	20	20
Hold	4	∞

8. Run the samples in the thermal cycler with the Ligation program.
9. Immediately proceed to Clean up ligation reaction.

Clean up ligation reaction

Note: Make sure the AMPure XP beads are equilibrated to room temperature before you begin.

1. Vortex the beads until the solution is homogeneous.
2. Add 48 µL of beads to each sample at room temperature (0.8X).
3. Vortex mix, then briefly centrifuge the sample plate.
4. Incubate the samples for 5 minutes at room temperature.
5. Place the sample plate on a magnet until the solution clears and a pellet has formed (~2 minutes).

6. Remove and discard the supernatant without disturbing the pellet (Less than 5 µL may be left behind.).
7. Add 180 µL of freshly prepared 80% ethanol solution to the sample while it is still on the magnetic plate. Do not disturb the pellet.
8. Incubate for 30 seconds, then carefully remove the ethanol solution.
9. Repeat steps 7 and 8 for a second ethanol wash.
10. Using a new pipette tip, remove any residual ethanol solution from the bottom of the well.
11. Allow beads to dry on the magnetic plate for 1–3 minutes.

    Note: Avoid over-drying beads, as this will reduce yield.

12. Add 20 µL of Lotus Elution Buffer to the sample plate.
13. Pipette mix until homogeneous.
14. Incubate at room temperature for 5 minutes.
15. Place the sample plate on a magnet for 2 minutes.
16. Transfer the supernatant containing your library to a new low-bind PCR plate, being careful to avoid any bead carryover.

    Safe stopping point: Libraries can be stored overnight at –20°C.

Perform PCR and Cleanup

1. Prepare the PCR Master Mix, adding the components in the order shown:

Reagent	Volume per sample (µL)
Lotus Elution Buffer	10
Lotus PCR Reagent	4
Lotus PCR Buffer	10
Lotus PCR Enzyme	1
Total volume: PCR Master Mix	**25**

   Note: Keep reagents and master mix on ice.

2. Gently vortex PCR Master Mix for 5 seconds.
3. Add 25 µL of the premixed PCR Master Mix to the entire eluted library sample (20 µL), then vortex mix (Total volume is 45 µL.).
4. Add 5 µL of UDI Primer mix to the PCR Master Mix (Total volume is 50 µL.).
5. Briefly centrifuge the sample plate, then put on ice.

6. Set up the thermal cycler with the PCR program as described with a heated lid set to 105°C.

Step	Cycles	Temperature (°C)	Duration (seconds)
Activate enzyme	1	98	30
Amplify	Varies, see Table 1	98	10
		60	30
		68	60
Hold	NA	4	∞

Important! The number of cycles required to produce sufficient library for sequencing will depend on sample input quantity and quality. In the case of low-quality samples, the number of cycles required may vary based on the quality of the sample and amount of usable DNA present. Approximate guidelines for high-quality DNA are indicated in Table 1, but the exact number of cycles required must be empirically determined. Adjust cycle # as needed.

Input material (ng)	Minimum PCR cycles
≥25	4
10	6
1	9

7. When the PCR program is complete, vortex the room-temperature AMPure XP beads until the solution is homogeneous.
8. Add 90 µL of AMPure XP beads (0.8X) to each sample well, and mix by pipetting.
9. Incubate the sample plate for 5 minutes at room temperature.
10. Place the sample plate on a magnet until the solution clears and a pellet has formed (~2 minutes).
11. Remove and discard the supernatant without disturbing the pellet (Less than 5 µL may be left behind.).
12. Keeping the sample plate on the magnet, add 180 µL of freshly prepared 80% ethanol solution without disturbing the pellet.
13. Incubate for 30 seconds, then carefully remove the ethanol solution.
14. Repeat steps 13 and 14 for a second ethanol wash.
15. With a new pipette tip, remove any residual ethanol solution from the bottom of the well.
16. Allow beads to dry on the magnet for 1–3 minutes.

    Note: Over-drying beads will reduce yield.

17. Add 22 µL of Lotus Elution Buffer to the sample wells, and mix by pipetting until homogeneous.
18. Incubate samples at room temperature for 5 minutes.
19. Place the sample plate on a magnet for 2 minutes.
20. Transfer the supernatant (20 µL) containing the final library to a new low-bind PCR plate. Be careful to avoid any bead carryover.

    Safe stopping point: Libraries can be stored overnight at –20°C.

Perform Purification

Note: We recommend performing this purification procedure to remove excess primers, thus minimizing adapter dimers and index hopping.

1. Vortex the AMPure XP beads until the solution is homogeneous.
2. Add 24 µL of beads to each sample well (1.2X).
3. Vortex mix, then briefly centrifuge the sample plate.
4. Incubate the plate for 5 minutes at room temperature.
5. Place the sample plate on a magnet until the solution clears and a pellet has formed (~2 minutes).
6. Remove and discard the supernatant without disturbing the pellet (Less than 5 µL may be left behind.).
7. Keeping the samples on the magnetic plate, add 180 µL of freshly prepared 80% ethanol solution.
8. Incubate for 30 seconds, then carefully remove the ethanol solution.
9. Repeat steps 7 and 8 for a second ethanol wash.
10. With a new pipette tip, remove any residual ethanol solution from the bottom of the well.
11. Allow beads to dry on the magnet for 1–3 minutes.

    Note: Over-drying beads will reduce yield.
12. Add 20 µL of Lotus Elution Buffer to the sample plate, then mix well by pipetting until homogeneous.
13. Incubate samples at room temperature for 5 minutes.
14. Place the sample plate on a magnet for 2 minutes at room temperature.
15. Transfer the supernatant containing the final library to a new low-bind PCR plate. Be careful to avoid any bead carryover.
16. Store freshly prepared libraries at 4˚C (or long term at –20˚C).

    The library is now ready for quantification, which can be performed using fluorometric methods (i.e., Qubit Fluorometer), or, for the highest accuracy, a qPCR based method can be used. A high-sensitivity TapeStation assay (or equivalent) can be used to ensure desired library size.

    Figure 3 shows an example of the final library product run on a TapeStation; the expected library size is ~350 bp when using the 200 bp fragmentation time.

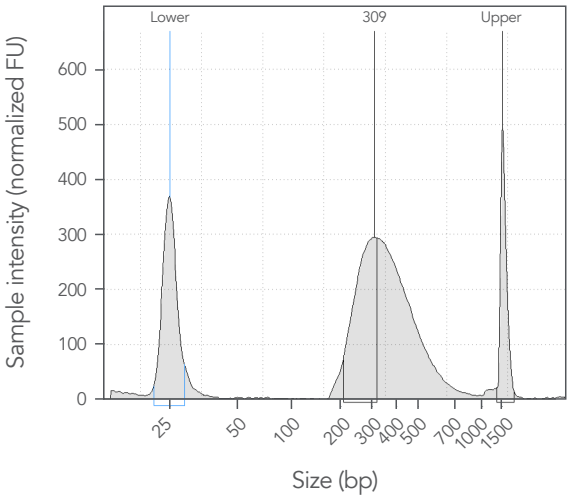

Figure 3. TapeStation trace of final library product.

SEQUENCING

Libraries are now ready for sequencing on an Illumina platform via the manufacturer's methods. Details are available at https://support.illumina.com/downloads/prepare_libraries_for_sequencing_miseq_15039740.html.

EXAMPLE DATA

This protocol was created using cDNA generated from contrived samples which were prepared by mixing inactivated SARS-CoV-2 viral material into negative background matrix of a negative nasopharyngeal (NP) swab in viral transport medium (VTM). Negative NP sample matrices were collected from healthy donors. A range of viral copies of the inactive virus were spiked into 200 µL of the negative matrix, and RNA was isolated using RNAdvance Viral (Beckman Coulter Life Sciences) following manufacturer recommendations. cDNA was generated using RNA with a C_t value of 29.3 and amplified according to this protocol. A Lotus cDNA library was prepared using 30 ng of pooled amplicons generated from primer sets 1 & 2. Below is the coverage of the SARS-CoV-2 genome with 2.1 million sequencing reads, and also sub-sampled to 40,000 reads. Reads that were shorter than 40 base pairs in length were removed prior to generating the coverage figures. 2 x 150 bp paired-end reads were generated on an Illumina MiSeq platform using a standard flowcell. With deeper sequencing, we were able to achieve 100X coverage for all positions within the genome (excluding the 5' and 3' UTR).

The data in Figure 4 demonstrate base coverage depth using 2.1 M reads per sample. Figure 5 shows base coverage depth after sub-sampling to 40,000 reads.

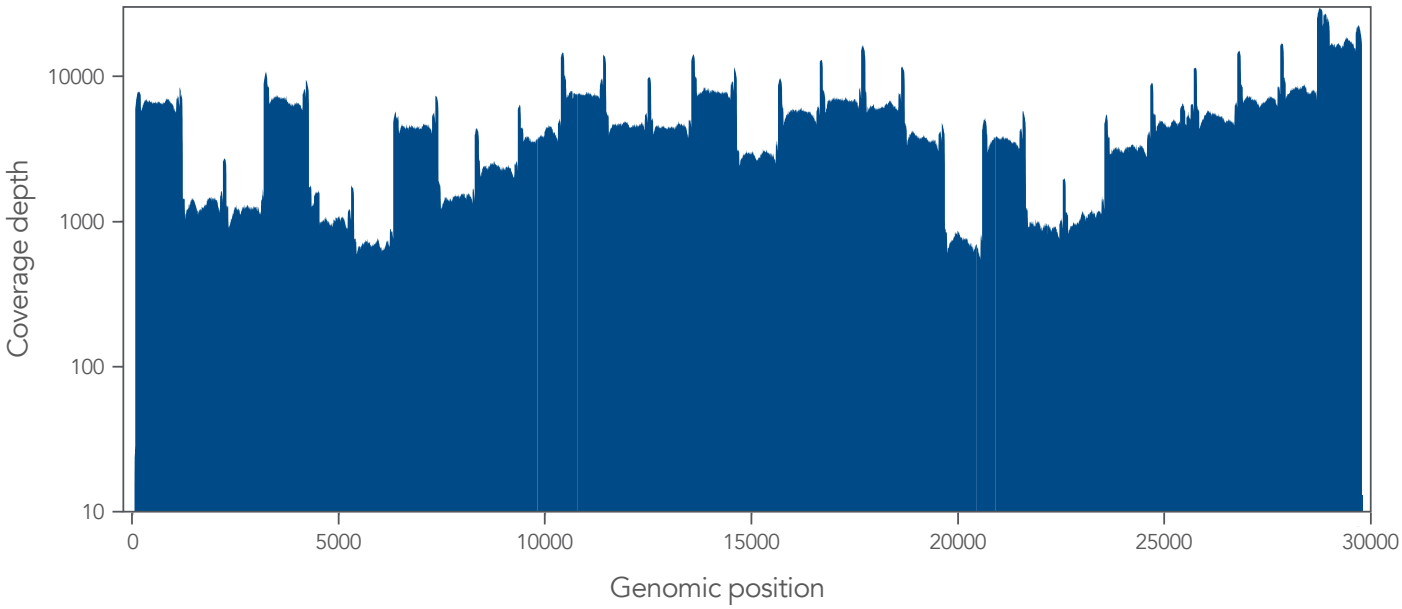

Figure 4. Data demonstrating base coverage depth using 2.1 M reads per sample.

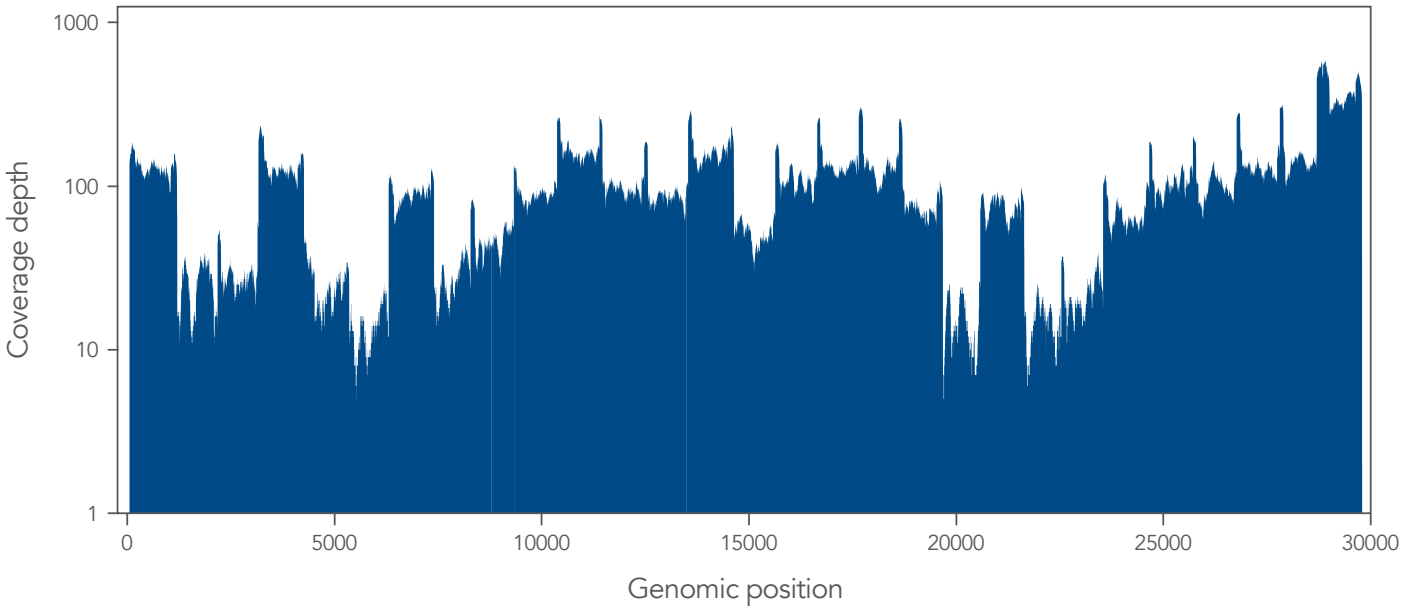

Figure 5. Base coverage depth after sub-sampling to 40,000 reads.

APPENDIX A

Note 1

In an independent experiment, we tried adding 6 µL of cDNA into the amplification reaction instead of 2.5 µL to investigate whether we would see an increase in amplified material; we found that the increase in cDNA volume in the amplification reaction did not increase final yield of the pools. Therefore, only amplicon pools generated using 2.5 µL of cDNA were carried through the entire protocol.

Note 2

As stated in the Amplicon Generation section, cycling parameters may need optimization. Here, we adjusted cycle number based on C_t value. Examples are shown in Data Table 1.

Data Table 1. Example data of RNA sample Ct values and corresponding post-amplification yields

C_t value based on N1 assay from 2019-nCoV RUO Kit	Number of PCR cycles for amplicon generation	Yield after clean-up (ng/µL)
29.3	30	~1.8
31.4	35	~9.8
33.2	35	~5.8

REFERENCES

1. Freed NE, Vlková M, et al. (2020) Rapid and inexpensive whole-genome sequencing of SARS-CoV-2 using 1200 bp tiled amplicons and Oxford Nanopore Rapid Barcoding. Biol Methods Protoc 5(1):bpaa014.
2. Freed N and Silander O (2020) nCoV-2019 sequencing protocol (RAPID barcoding, 1200bp amplicon) v4 (protocols.io.bh7hj9j6). protocolsio.

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