Sequencing SARS-CoV-2
With the ARTIC nCoV-2019 Amplicon Panel

This protocol uses:

- xGen™ DNA Library Prep MC Kit

INTRODUCTION

The method presented here is provided by IDT. This method can be used as a starting point for using the ARTIC nCoV-2019 Amplicon Panel and the xGen DNA Library Prep MC Kit in similar experiments but has not been tested for other applications. 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.

OVERVIEW

Sequencing viral samples has been made more straightforward by amplification enrichment approaches that are compatible with total RNA from samples that include virus and human cells. In these experiments, 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 used to separate overlapping amplicons. After amplification, the DNA is pooled and ligated to form an NGS-compatible library. Two sequencing platforms from Oxford Nanopore Technologies® and Illumina®, are in common use for this process.

The ARTIC nCov-2019 Amplicon Panel was created by Dr. Josh Quick of Birmingham University in the United Kingdom [1]. This panel consists of two pools of primers that produce amplicons of approximately 400 bp in length. This protocol describes the process for creating libraries that are designed for use on the Oxford Nanopore® sequencing platform; however, access to this technology can be limiting. Here, we present the use of the xGen DNA Library Prep MC Kit as a workflow (Figure 1) to adapt the ARTIC Amplicon Panel for sequencing with Illumina® sequencers. This protocol describes the process for creating libraries that can be used for sequencing intact non-fragmented amplicons, which simplifies downstream sequence analysis and reduces data loss due to primer trimming.

Note: This method is compatible with the xGen Normalase™ Module (see Appendix A: Perform Normalase Reaction) for instructions before starting your PCR setup.

1	cDNA synthesis	30 minutes
2	Amplicon generation	3.5 hours
3	Post-amplification cleanup	0.8X SPRIselect • 20 minutes
4	End-repair/A-tail	60 minutes
5	Adapter Ligation	15 minutes
6	Post-ligation cleanup	0.8X SPRIselect • 20 minutes
7	Indexing PCR	30 minutes
8	Post-PCR cleanup	0.65X SPRIselect • 20 minutes

Figure 1. Overview of the ARTIC nCov-2019 Amplicon Panel workflow.

CONSUMABLES AND EQUIPMENT

Table 1. Consumables from IDT

Workflow component	Product name	Index number	Reaction size (rxn)	Catalog number
xGen core reagents	xGen DNA Library Prep MC Kit	N/A	16	10009861
	xGen DNA Library Prep MC Kit	N/A	96	10009819
Amplicon panel	ARTIC nCoV-2019 Amplicon Panel		500	website
xGen Normalase Module (optional)	xGen Normalase Module	N/A	96	10009793
xGen CDI Primers	xGen CDI Primers	D501-D508/ D701-D712	96	10009815
xGen Normalase CDI Primers	xGen Normalase CDI Primers	D501N-D508N/ D701N-D712N	96	10009794
xGen Normalase UDI Primer Plates	xGen Normalase UDI Primer Plate 1	SU001-SU096	96	10009796
	xGen Normalase UDI Primer Plate 2	SU097-SU192	96	10009797
	xGen Normalase UDI Primer Plate 3	SU193-SU288	96	10009798
	xGen Normalase UDI Primer Plate 4	SU289-SU384	96	10009799
	xGen Normalase UDI Primer Set 1	SU001-SU384	4 x 96	10009795
	xGen Normalase UDI Primer Set 2	SU385-SU768	4 x 96	10009800
	xGen Normalase UDI Primer Set 3	SU769-SU1152	4 x 96	10009811
	xGen Normalase UDI Primer Set 4	SU1153-SU1536	4 x 96	10009812
Accessories	Nuclease-Free Water	N/A	N/A	11-04-02-01

Table 2. Consumables from other suppliers

Item	Suppler	Catalog #
Q5® High-Fidelity 2X Master Mix	New England Biolabs	M0492S (100 rxn)
Absolute ethanol (200 proof)	General laboratory supplier	Varies
SPRIselect® or AMPure® XP beads, or equivalent	Beckman Coulter	B23317/B23318/ B23319 or A63880/A63881/ A63882
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	032851 or 032854
High Sensitivity DNA Kit, or equivalent	Agilent	5067-4626
High Sensitivity D1000 ScreenTape® or equivalent	Agilent	5067-5584
0.2 mL PCR tubes	Various suppliers	Varies
Aerosol resistant tips and pipette ranging from 1–1000 µL	Various suppliers	Varies
Invitrogen™ SuperScript™ IV Reverse Transcriptase, or similar	Thermo Fisher Scientific	18-090-050

Table 3. Equipment

Item	Suppler	Catalog #
2200 TapeStation/4200 TapeStation System or equivalent	Agilent	G2965AA or G2991AA
Qubit™ 4 Fluorometer, or equivalent	Thermo Fisher Scientific	033226
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 Scientific	033226
0.2 mM magnets for tubes and plates	Permagen	MSR812/MSP750
Vortex	General laboratory supplier	Varies

TEMPLATE GENERATION

cDNA synthesis

The ARTIC nCoV-2019 Amplicon Panel supports cDNA as input. Select a cDNA synthesis module [2] that supports 2 step RT- PCR, includes random primers, and has a processivity of >1 kb. This protocol has been tested with SARS-CoV-2 genomes isolated from nasopharyngeal swabs. Extracted RNA was used as input into the Superscript® IV First-Strand Synthesis System (Thermo Fisher Scientific). The manufacturer's protocol was followed as written using the random primers and associated specifications. Optional host gDNA/RNA removal steps were not performed. Further, the optional RNase H step was not performed, and the resulting cDNA was used directly as input into the ARTIC Amplicon Panel.

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

Important: There is no cleanup step prior to amplicon generation.

Amplicon generation

Important: Primer pools 1 & 2 are intended to be used separately during amplicon generation [1, 2] and should not be combined. Each primer pool will need to be diluted before use.

1. Prepare primer pools 1 & 2 by diluting the ARTIC Primer Pools 1:10 in IDTE, pH 8.
2. Mix, then briefly centrifuge all components before use.
3. Prepare the Amplicon Master Mix as follows (one mix per primer pool):

Reagent	Volume per sample (µL)
Q5 Hot Start Master Mix (New England Biolabs)	12.5
Diluted ARTIC Primer Pool 1	3.6
Nuclease-Free Water	2.9
Total volume	19

Reagent	Volume per sample (µL)
Q5 Hot Start Master Mix	12.5
Diluted ARTIC Primer Pool 2	3.6
Nuclease-Free Water	2.9
Total volume	19

4. Combine and mix the reagents thoroughly to create the Amplicon Master Mix.
5. Add 6 µL of cDNA to the appropriate wells of a low-bind PCR plate or tube (See Appendix A). Each sample will need two wells (one well per primer pool).
6. Add 19 µL of Amplicon Master Mix to the appropriate sample.
7. Seal, briefly vortex, and centrifuge sample.

8. Place sample on a thermal cycler and run the following program:

Step	Temperature (°C)	Duration	Cycles
Heat activation	98	30 seconds	
Denaturation	95	15 seconds	35
Annealing and extension	63	5 minutes	
Hold	4	∞	

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

9. Allow SPRIselect® beads (Beckman Coulter) to equilibrate to room temperature. Vortex until homogenous before use.
10. Briefly centrifuge the amplified cDNA sample.
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 or tube.
12. Add 40 µL (0.8X) of SPRIselect beads per sample.
13. Mix well by pipetting.
14. Incubate at room temperature for 5 minutes.
15. Transfer sample to a magnet and allow beads to form a pellet (approximately 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. Be careful to not disturb the pellet.
18. Repeat the previous wash step for a total of 2 washes.
19. Using a fresh p20 pipette tip, remove any residual ethanol solution from the bottom of the well.
20. Remove sample from the magnet and add 22 µL of Nuclease-Free Water, then resuspend by mixing.
21. Incubate at room temperature for 3 minutes.
22. Transfer the sample back to the magnet and allow beads to collect for 5 minutes.
23. Carefully transfer 20 µL of supernatant to a new low-bind PCR plate or tube, being careful to not disturb the pellet.

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

Important: Measure the concentration of the amplicon pool using a Qubit™ fluorometer or equivalent, to ensure a measurable input (recommended 10 ng) goes into the library preparation.

LIBRARY PREPARATION

This library preparation kit has been designed for use with a wide range of DNA inputs: 1 ng to1 µg.

Prepare reagents

1. Upon receipt, store the reagents at –20 °C, except for Low EDTA TE, which is stored at room temperature.
2. Place all kit enzymes on ice (not in a cryocooler) for at least 10 minutes to allow enzymes to reach 4°C before pipetting.
3. After thawing reagents, briefly vortex (except the enzymes) to mix well.
4. Spin all tubes in a microcentrifuge to collect contents before opening.
5. Assemble all reagent master mixes ON ICE and scale volumes as appropriate, using 5% excess volume to compensate for pipetting loss. Always add reagents to the master mix in the specified order as stated throughout the protocol.
6. 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.

Avoid cross contamination

To reduce the risk of DNA and library contamination, physically separate the laboratory space, equipment, and supplies where pre-PCR and post-PCR processes are performed. Follow the instructions below to avoid cross-contamination:

1. Clean lab areas using 0.5% sodium hypochlorite (10% bleach).
2. Use barrier pipette tips to avoid exposure to potential contaminants.
3. Always change tips between each sample.

Perform end preparation

1. Transfer the amplicon sample to a sterile 0.2 mL PCR tube and adjust the volume of the sample to a final volume of 50 µL using Low EDTA TE, if necessary.
2. Preset a thermal cycler according to the program listed below with the heated lid set to 70°C (a heated lid is required):

Step	Temperature (°C)	Duration
Hold	4	∞
Enzymatic Prep	20	30 minutes
	65	30 minutes
Hold	4	∞

3. Prepare the End-Prep Master Mix in the order listed in the table:

Reagent	Volume per sample (µL)
Low EDTA TE	3
Buffer E1	4.7
Enzyme E2	1
Enzyme E3	1
Enzyme E4	0.3
Total Master Mix	**10**
DNA	50
Total Volume	**60**

4. Thoroughly mix the Master Mix by moderate vortexing for 5 seconds.
5. Add 10 µL of pre-mixed Master Mix to each sample and mix thoroughly by moderate vortexing for 5 seconds.
6. Spin down the sample tubes in a microcentrifuge and place them in the chilled thermal cycler and advance the program to the 20°C step.
7. Before the thermal cycler program completes, begin to prepare your master mix for the adapter ligation step. The samples should be kept at 4°C for no more than one hour.

Perform adapter ligation

1. Preset a thermal cycler program for 15 minutes at 20°C with lid heating OFF.

   Important: When using DNA input <50 ng, Reagent L3 (stubby Y adapter) must be diluted in Low-EDTA TE buffer to the appropriate concentrations, as outlined in the table below. Adapter dilution is necessary to reduce levels of adapter dimer formation. For certain applications, adapter dilution may need to be adjusted to achieve the results necessary to obtain sufficient sequencing data.

DNA input (ng)	Adapter
> 50	No dilution
10	10-fold (1:10)
1	20-fold (1:20)

   For intermediate inputs scale the adapter dilution accordingly. Testing may be required to determine the conditions best for your application.

2. Refer to the table below for preparing the Ligation Master Mix when using a stubby Y adapter (Reagent L3).

Reagents	Volume per sample (µL)
Buffer L1	18
Enzyme L2	6
Reagent L3*	5
Total Master Mix	**29**
DNA sample	60
Total Volume	**89**

* The Ligation Master Mix without the truncated adapter (Reagent L3) can be prepared ahead of time and kept on ice when making the End Prep Master Mix. Add the adapter to the Master Mix just before use.

3. Add the pre-mixed Ligation Master Mix (29 µL) directly to the End Prep reaction mixture.
4. Thoroughly mix the Ligation reactions by moderate vortexing for 5 seconds and spin down the sample tubes in a microfuge.
5. Place in the thermocycler, programmed at 20°C for 15 minutes with lid heating OFF.

Perform cleanup

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

1. Vortex the bead mixture until the solution is homogeneous.
2. Add 72 µL (0.8X) of SPRIselect beads to each sample at room temperature.
3. Vortex to mix, then briefly centrifuge the sample.
4. Incubate the samples for 5 minutes at room temperature.
5. Place the sample 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 magnet. 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. Add 20 µL of IDTE Buffer to the sample.
12. Pipette to mix until homogeneous.
13. Incubate at room temperature for 5 minutes.
14. Place the sample on a magnet for 2 minutes.
15. Transfer the supernatant containing your library to a new low-bind PCR tube/plate, being careful to avoid any bead carryover.

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

    PCR-free libraries are now ready for quantification, which should be performed by qPCR. Note that PCR-free libraries cannot be accurately quantified or assessed for library size by electrophoretic methods.

    Important: A second clean-up (using a 0.8X or a different bead ratio) may be beneficial when PCR-free libraries are prepared for direct sequencing on patterned flow cells with the potential for index hopping. If using unique dual indexed adapters, this additional cleanup is not necessary.

Perform indexing PCR

The ligation step is followed by an Indexing PCR step when using xGen stubby Y adapters (Reagent L3 supplied in xGen DNA Library Prep MC Kit).

Use the HiFi Polymerase Master Mix supplied in the kit for direct sequencing.

DNA Input	Minimum recommended cycles
1 µg	3*
100 ng	3*
10 ng	6–7
1 ng	9–10

* When indexing by PCR, a minimum of 3 cycles is required to complete adapter sequences, irrespective of whether enough library is available following ligation.

1. Preprogram the following thermal cycler program, adjusting the number of cycles depending on the input amount and workflow (see previous table).

Step	Temperature (°C)	Duration
Melting	98	2 minutes
PCR Cycles	98	20 seconds
	60	30 seconds
	72	30 seconds
Final Extension	72	1 minute
Hold	4	∞

2. Indexing PCR: add indexing primers directly to the eluted library following the table below as a guideline. Add 25 µL PCR Master Mix to each sample and mix by moderate vortexing for 5 seconds and spin down in a microcentrifuge.

   Note: If using xGen Normalase Indexing primer pairs, see Appendix A and the xGen Normalase Module Protocol for specific instructions.

Indexing options	Reagents	Volume per sample (µL)
xGen UDI primer pairs	Premixed primer pair	5.0
xGen CDI primer pairs	i5 primer	2.5
	i7 primer	2.5

Reagents	Volume per sample (µL)
PCR Master Mix	25
Sample + Primer Mix	25
Total Volume	**50**

3. Place in the thermal cycler and run the program.

Perform post-PCR cleanup

1. Purify the PCR amplification reaction using a SPRI bead suspension, magnetic rack, and freshly prepared 80% ethanol. Invert or briefly vortex beads to homogenize the suspension before use.
2. Add 32.5 µL SPRI beads (0.65 ratio) to each sample. Mix by pipetting 10 times, or until homogenous. Make sure, there are no bead-sample suspension droplets left on the sides of the tube.
3. Incubate the samples for 5 minutes at room temperature. Pulse-spin the samples in a microcentrifuge. Place the sample tubes on a magnetic rack until the solution clears and a pellet is formed (~2 minutes).
4. Remove and discard the supernatant without disturbing the pellet.
5. Add 200 µL of freshly prepared 80% ethanol solution to the pellet while it is still on the magnet. Be careful to not disturb the pellet
6. Incubate for 30 seconds, then carefully remove the ethanol solution.
7. Repeat step 6 once for a second wash with the ethanol solution.
8. Pulse-spin the samples in a microcentrifuge, place back onto the magnet, and remove any residual ethanol solution from the bottom of the tube.
9. Add 20 µL of IDTE Buffer to the sample.
10. Pipette to mix until homogeneous.
11. Incubate at room temperature for 5 minutes.
12. Place the sample on a magnet for 2 minutes.
13. Transfer 20 µL eluate volume to a new 0.2 mL PCR tube. Ensure that the eluate does not contain magnetic beads (indicated by brown coloration in eluate). If magnetic beads are present, place back on magnet, and transfer eluate again.

Important: If direct sequencing on patterned flow cells, perform the following second cleanup as described in Appendix A: Perform Normalase Reaction to ensure optimal removal of the unincorporated primers which can increase index hopping on patterned flow cells.

Note: A second purification is not necessary if using xGen Unique Dual Indexing primer pairs/adapters, or if using the xGen Normalase Module or xGen Hybridization Capture.

Perform library quantification

The library is now ready for quantification, which can be performed using fluorometric methods (e.g., Qubit™) or qPCR. An electrophoretic chip (i.e., High Sensitivity DNA Agilent Bioanalyzer kit) can be used to ensure desired library size and confirm quantification. Store libraries at –20 °C.

Library sequencing recommendations

Due to the amplicon size generated by the ARTIC v4 primers (~400 bp), it is recommended to sequence using 2 x 250 bp PE sequencing. This read length should fully span the amplicons, leading to more complete genomic coverage.

APPENDIX

Appendix A: Perform Normalase reaction

Review this section and the **xGen Normalase Module protocol** before setting up your Normalase PCR. To achieve the expected results, amplify each library using xGen Normalase Primer Pairs with the appropriate number of cycles and thermocycling conditions shown here to obtain a library yield of 12 nM or greater in a 20 µL eluate.

1. To each sample, add 2 µL of each of the Normalase CDI Primers or 4 µL of Normalase UDI Primer Pairs for a total volume of 24 µL.

Indexing options	Minimum recommended cycles	Volume per sample (µL)
xGen Normalase UDI Primer Pairs	Premixed primer pair	4
xGen Normalase CDI Primer Pairs	i5 primer	2
	i7 primer	2

2. Assemble the PCR Master Mix on ice according to the table below. Mix thoroughly and pulse-spin to collect contents. Add 26 µL of the mix to each sample tube, mix thoroughly, and pulse-spin to collect contents (50 µL total reaction volume):

Component	Volume per reaction (µL)
PCR Master Mix	25
Reagent R7	1
Total Master Mix	**26**
Eluted Sample + Primers	24
Total Volume	**50**

3. Run the following thermal cycler program, adjusting the number of cycles depending on the input amount and sample quality (see table below) (Lid heating set to ON):

Step	Temperature (°C)	Duration
Melting	98	2 minutes
PCR Cycles	98	20 seconds
	60	30 seconds
	72	30 seconds
Final Extension	72	5 minutes
Hold	4	∞

4. Proceed to **Perform post-PCR cleanup** section.
5. Proceed to Normalase I, pooling, and Normalase II in the **xGen Normalase Module protocol**.

Appendix B: Primer sequences

The primer sequences are listed here for your reference; these primers include full-length Illumina® adapter and index sequences.

- **i7 primer:** Replace 8 (CDI) or 10 (UDI) X's with the **REVERSE COMPLEMENT** of the specified i7 index sequence in the Index Sequences Master List:

  5´ – CAAGCAGAAGACGGCATACGAGAT**XXXXXXXX(XX)**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT – 3´

- **i5 primer:** Replace 8 (CDI) or 10 (UDI) Y's with the specified Forward Strand Workflow i5 index sequence in the Index Sequences Master List:

  5´ – AATGATACGGCGACCACCGAGATCTACAC**YYYYYYYYYY**ACACTCTTTCCCTACACGACGCTCTTCCGATCT – 3´

Tip: If you would like assistance confirming compatibility of your own primers with the xGen Amplicon workflow, contact applicationsupport@idtdna.com. To purchase custom Normalase Indexing Primers, contact your local sales representative or distributor.

If you need additional technical support, contact IDT at applicationsupport@idtdna.com.

REFERENCES

1. Quick J. nCoV-2019 sequencing protocol v3 (LoCost) V.3. Protocols.io. Accessed March 15, 2022.
2. Rajan D, Betteridge E, Shirley L. et.al. COVID-19 ARTIC v3 Illumina library construction and sequencing protocol. Protocols.io. Accessed March 15, 2022.

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