TITLE: **Amplification of SARS-COV2 RNA Using Artic V4 Primers**		PAGE: 1 of 10
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1. PURPOSE

To amplify extracted SARS-CoV2 RNA for the purpose of sequencing on an Illumina platform.

*This protocol has been adapted from the xGen DNA Library Prep Artic V4 SARS-CoV2 protocol, which can be found here: https://www.idtdna.com/pages/products/next-generation-sequencing/workflow/xgen-ngs-amplicon-sequencing/predesigned-amplicon-panels/artic-sc2-amp-panel#product-details.

2. IMPORTANT MANUALS/PROTOCOLS

One Step Ahead RT-PCR (Qiagen)
Illumina DNA Library Prep Kit & Indices

3. REQUIREMENTS

3.1 MATERIALS

Amplification
One Step Ahead RT-PCR System (Qiagen: 220213)
Artic V4.1 NCOV-2019 Panel (IDT 10011442)
Nuclease-free water (e.g. Ambion nuclease-free water)
RNase-free microcentrifuge tubes (preferably LoBind or equivalent)
RNase-free PCR tubes/plates (e.g. Bio-Rad HSS9601)
PCR seals (BioRad: MSB1001, MSA5001)
RNase-free filter pipette tips (preferably low-retention)
Ethanol (100%, molecular biology grade)

Library Prep
Illumina DNA Library Prep Kit (Illumina: 20060059)
IDT For Illumina DNA/RNA UD Indices (Illumina: 20027213)
50 mL reagent reservoirs (e.g. 229290)
AMPure XP beads (Beckman Coulter A63880)
SPRIselect beads (Beckman Coulter B23318)

Qubit dsDNA HS Assay Kit (Thermo Fisher: Q32854)
96-well microplate for fluorescence assays (Thermo Fisher: M33089)

3.2 EQUIPMENT

Magnetic stand (e.g. Thermo Fisher: AM10027, NEB: S1509S)
Electronic pipettes (P12.5, P125, P1000)
P200 manual multichannel pipette
Qubit fluorometer (Thermo Fisher)
Microplate centrifuge
TapeStation/Bioanalyzer (Agilent)
PX1 PCR plate sealer (Bio-Rad)

4. NOTES

Operations

- RNA extraction must be performed prior to the start of this protocol. Extraction of SARS-CoV2 RNA must be carried out under BSL-2+ conditions.
- All operations should take place in designated “pre-” and “post-PCR” areas in order to minimize contamination of RNA samples with PCR products. Physical separation of these areas and their regular decontamination should be sufficient, but including a negative extraction control in each sample set is the best practice.

Primers

All primers may be synthesized and purified under standard conditions, but freeze-thaw cycles should be minimized for both stocks and working solutions. We always order the standard desalted primers from Integrated DNA Technologies (IDT) as we have encountered low quality issues with primers from some other suppliers.

The primer set for pool A and pool B samples can be found here: https://github.com/artic-network/artic-ncov2019/blob/master/primer_schemes/nCoV-2019/V4/SARS-CoV-2.primer.bed

Sequencing

- We have routinely achieved sufficient coverage for ~50-60 samples on the MiSeq platform using V3 chemistry for 2x300 paired-end reads and up to 384 samples on the Nextseq 500 using V2.5 High Output 2x150 paired-end reads.

5. PROTOCOL

5.1 M-RTPCR Amplification

Prepare a new primer pool fresh before starting the PCR reactions for best efficiency and to avoid primer dimers. Each primer pool should be created and kept separately, carefully avoiding contamination of any pool A primers in pool B and vice versa.

Primer pools A and B should contain all primers from each pool (50 sets, 100 primers in total), pooled at equal volumes for final concentration of 10µM. Scale primer pools accordingly to accommodate all samples.

Each mRT-PCR reaction will undergo separate library prep. The reactions for each sample will not be merged until after trimming of the primers during data analysis.

5.2 SARS-CoV2 M-RTPCR part 1: cDNA synthesis and amplicon generation

1. Thaw RNA samples on ice. 10µM primer pools may be thawed at room temperature, vortexed, and placed on ice.
2. Prepare master mix for RT-PCR on ice for 5µL volumes of RNA based on the below quantities of reagents, which are for one sample reaction. Enzyme should be added last and kept on a cold block throughout the setup.

mRT-PCR – pool A reaction	**mRT-PCR – pool B reaction**
4µL Rnase-free water	4µL Rnase-free water
10µL 2.5X RT-PCR Master Mix	10µL 2.5X RT-PCR Master Mix
5µL 10µM primer pool A	5µL 10µM primer pool B
1µL OneStep Ahead RT Mix	1µL OneStep Ahead RT Mix
= 20µL total	= 20µL total

3. Add 20µL master mix to each tube/well and add 5µL RNA sample or water negative control to each volume. Mix by pipetting and spin down briefly before placing in a thermal cycler pre-warmed to 45°C with a heated lid offset of 10°C.
4. 4-stage cycling parameters:
   Pool A:
   a. 45°C for 15'; 95°C for 5'
   b. [95°C for 15s; 52°C for 30s; 68°C for 8'] x 12 cycles
   c. [95°C for 15s; 52°C for 15s; 68°C for 2'30s] x 35 cycles
   d. 72°C for 5'; 4°C hold
   Pool B:
   a. 45°C for 15'; 95°C for 5'
   b. [95°C for 15s; 55°C for 30s; 68°C for 8'] x 12 cycles
   c. [95°C for 15s; 55°C for 15s; 68°C for 2'30s] x 35 cycles
   d. 72°C for 5'; 4°C hold

5. Run 3µL of each reaction on a 0.8% agarose gel to confirm M-RTPCR amplification (~400 bp amplicons). Amplicons should be stored at -20°C for long-term use or can be kept at 4°C for several days before proceeding to library preparation

5.3 Sample Purification and Library Preparation

5.3.1 Initial M-RTPCR Amplicon Cleanup

- Bring the volume of each PCR reaction to 50µL with dH_2O
- Allow AMPure XP beads to warm to room temperature before thoroughly resuspending beads by vortexing for 1'. During this time prepare a fresh volume of 80% ethanol for bead washing.
- Add 0.9x beads by volume (i.e. 45µL) to each sample, vortexing as necessary to keep beads in solution. Pipette each sample thoroughly and incubate at room temperature for 5'.
- Place samples on the appropriately sized magnetic rack until beads clump on the side of each tube/well, approximately 2-3'. Remove and discard supernatant containing undesired small fragments.

- Add 200µL 80% ethanol to wash each sample with a P200 manual multichannel pipette and incubate for 30s on the rack. Remove total volume of supernatant and discard. Don't resuspend the beads.
- Repeat Step 5, this time carefully removing the full volume of wash solution first with a P200 manual multichannel pipette and next with a P12.5 multichannel electronic pipette at the bottom of each well to minimize ethanol carryover. Allow each sample to air-dry for 3-5' on the rack, just until the surface of each bead mass is no longer glossy.
- Working quickly, remove the samples from the rack and add 32µL nuclease-free water to each well. Resuspend the entire bead mass by pipetting and incubate at room temperature for 5'.
- Place samples back on the magnetic rack until beads clump and the supernatant is clear, approximately 3'. Remove 30µL to a clean, labeled LoBind tube or PCR plate for storage, minimizing bead carryover.

5.3.2 Quantitation and Dilution

Following successful amplification and purification, one can proceed with the next steps for input into the Illumina protocol:

1. Perform readings of samples using the Qubit dsDNA High Sensitivity assay with fresh standards. Sample readings should allow for consistent dilution if necessary to the required 50-250 ng/µL for consistent Illumina tagmentation.
2. Proceed to Illumina library prep as soon as possible to avoid fluctuations in concentration of normalized samples. Freeze-thaw should be avoided and for delays > 24 hours, sample concentration should be confirmed again by Qubit.

5.3.3 Illumina DNA Preparation

The following protocol is written for high-throughput library preparation using multichannel pipettes and a 96-well plate with *n* samples, *r* rows, and *c* columns. For DNA inputs between 50-250 ng/µL, quantifying and normalizing the initial DNA sample is not required.

This protocol is adapted from the Illumina DNA Prep Reference Guide (1000000025416).

Tagment Genomic DNA

1. Thoroughly clean working area and pipettes with 10% (v/v) bleach or equivalent solution followed by 70% ethanol in order to avoid contamination of samples.
2. Remove Bead-Linked Transposomes (BLT) from the 4°C fridge and Tagmentation Buffer 1 (TB1) from the -20°C freezer and thaw at RT. Gently vortex and spin down both reagents before using.
3. Make the Tagmentation Master Mix (TMM) by mixing *n* x 1.02 x 5.5 µL BLT and *n* x 1.02 x 5.5 µL of TB1 in a LoBind tube. Mix thoroughly by vortexing.
4. Distribute TMM into *r* PCR strip tubes with *c* x 1.02 x 10.0µL in each tube.
5. With a P12.5 pipette, distribute 10µL TMM into all appropriate wells of a fresh PCR plate (the "tagmentation plate").
6. With a P125 multichannel pipette, transfer 15 µL of cDNA (50-250 ng/µL) into the tagmentation plate for a total volume of 25µL per well. Mix gently by pipetting up and down 10 times with the same multichannel pipette, changing tips after every transfer.
7. Heat seal the plate.
8. Spin plate briefly to collect reaction volume at the bottom of each well (e.g. 1000g for 1').
9. Place the plate in a pre-heated thermal cycler and run the following program with a heated lid:
   - 55°C for 15'
   - Hold at 10°C

   (Nb: be sure that the thermal cycler lid is tightly sealed.)
10. During the incubation, remove the TSB from the 4°C fridge
11. Remove tagmentation plate from the thermal cycler as soon as the 15' at 55°C ends, spin briefly to collect condensation.

Post Tagmentation Cleanup

1. Before starting, prepare fresh 80% EtOH from absolute ethanol
2. Add 5 µL TSB to each well in the tagmentation plate, slowly pipetting 10X to mix. Use *r* PCR strip tubes for easy addition. Spin briefly and incubate for 5' at RT, then keep on ice or at 4°C until needed.
3. Place the plate in a pre-heated thermal cycler and run the following program with a heated lid:
   - 37°C for 15'
   - Hold at 10°C

(Nb: be sure that the thermal cycler lid is tightly sealed.)

4. Remove plate from the thermal cycler as soon as the 15' at 37°C ends.
5. Place plate on magnetic stand for 3', remove supernatant
6. Wash 2X with 80% EtOH
7. Add 50 µL Tagmentation Wash Buffer (TWB) directly onto the beads. Ensure that the plate is off the magnetic stand when adding TWB. Pipette slowly until beads are fully resuspended.
8. Place the plate on magnetic stand for 3'
9. Remove supernatant (50 µL)
10. Add 100 µL TWB directly onto the beads. Pipette slowly until beads are fully resuspended.
11. Heat seal the plate and place on the magnetic stand for 3' until the liquid is clear
12. Keep on the magnetic stand until the next step. The TWB (50 µL) remains in the wells to prevent overdrying of the beads.

PCR Reaction

1. Before starting, bring out the Enhanced PCR Mix (EPM) from the -20 freezer. Allow it to thaw at RT before use. Also, bring out IDT for Illumina DNA/RNA UD Indexes from the -20°C freezer and thaw at RT.
2. Gently vortex and centrifuge the EPM before using.
3. Create PCR master mix by adding *n* x 1.02 x 11 µL EPM and *n* x 1.02 x 11 µL nuclease-free water. Vortex for 10s and centrifuge before use.
4. With plate on the magnetic stand, remove 50 µL supernatant. Any foam that remains on the well walls will not adversely affect the library.
5. Remove the plate from the magnet and immediately add 20 µL PCR master mix to each sample well.
6. Pipette gently 10X to mix until the beads are fully resuspended.
7. Gently vortex and centrifuge the UD index plates. For pool A and pool B primers, use different index plates.
8. Add 5 µL of index adapters from the UD index plate to each well. Take note of which plates and rows you have used.
9. Using a pipette set to 20 µL, pipette 10X to mix. Alternatively, seal the plate and use a plate shaker at 1600 rpm for 1'.
10. Heat seal the plate and centrifuge at 3000 rpm for 1'
11. Place the plate in a pre-heated thermal cycler and run the following program with a heated lid:
    - 68°C for 3'

- 98°C for 3'
- [98°C for 45s; 62°C for 30s; 68°C for 2'] x 5 cycles
- 68°C for 1'
- 10°C hold

12. When complete, remove plate from the thermal cycler, spin briefly to collect condensation, and place on ice if continuing to next step immediately or store in the 4°C fridge. This is a safe stopping point.

5.4 Clean-Up Libraries, Pool Libraries, and Perform Size Selection

Library Clean-Up

1. Prepare fresh 80% EtOH from absolute ethanol
2. Bring out Resuspension Buffer (RSB) from the -20°C freezer and thaw at RT
3. Place the plate on the magnetic stand for 5'
4. Transfer 22.5 µL of supernatant from each well of the PCR plate to a new PCR plate
5. Vortex for 1' and invert SPRIselect beads multiple times to resuspend
6. Add 22.5 µL nuclease-free water into each well, so the total volume in each well is 45 µL
7. Add 81 µL SPRIselect Beads to each well containing supernatant (1.8x ratio). Pipette each well 10x to mix.
8. Incubate the sealed plate at RT for 5'
9. Place plate on magnetic stand for 5' and wait until the liquid is clear
10. Without disturbing the beads, remove and discard supernatant (~ 126 µL)
11. Wash two times as follows:

a. With the plate on the magnetic stand, add 200 µL fresh 80% EtOH without mixing
b. Incubate for 30s
c. Without disturbing the beads, remove and discard supernatant

12. Use a 20 µL pipette to remove and discard residual EtOH
13. Air-dry plate on magnetic stand for 2'
14. Remove plate from the magnetic stand
15. Add 32 µL RSB to the beads and pipette 10X to resuspend
16. Incubate at RT for 5'
17. Place the plate on the magnetic stand and wait until the liquid is clear (5')
18. Transfer 30 µL supernatant to a new 96-well PCR plate

Safe stopping point: store at -25°C to -15°C for up to 30 days

19. Perform readings of samples using the Qubit dsDNA High Sensitivity assay with fresh standards to determine post-amplification concentration.
20. Use Tapestation (D1000, HS) to check library quality and fragment size (2µL sample or ladder, 2µL buffer).
21. If both size and concentrations look sufficient, proceed to pooling of libraries

Pooling of Libraries
1. Libraries should be pooled at equal molarity. Input both the average fragment size of the library and the concentration as calculated by Qubit in the nM calculator spreadsheet to calculate individual molarities for each library. Determine the minimum concentration needed in order to pool the samples equimolarly.
2. Calculate and add the appropriate amount of nuclease-free water to samples in order to dilute to the minimum concentration. Mix well by pipetting.
3. Wait until the liquid is clear and combine equal volumes of each sample into one tube. Vortex to mix.

Size Selection
Complete size selection using SPRIselect beads.

Before beginning, prepare three 1mL tubes of 85% ethanol.

Left side size selection:

- 0.75 X ratio (µL DNA pooled)

1. Add the appropriate amount of beads directly into the LoBind tube containing the pooled DNA
2. Vortex for 1' (or pipet 10 times), RT for 2', place microtube on magnetic rack for 5'
3. Remove supernatant
4. Take microtube off of the magnetic rack
5. Wash with 1 mL of 85% ethanol (use electronic P1000 pipette)
6. RT for 1'
7. Repeat the ethanol wash (repeat steps 6 and 7)
8. Use P12.5 electronic pipette to remove 10µL from the bottom of the microtube
9. Air dry at room temperature for 5', make sure microtube cap is open
10. Elute with 52 µL water, RT 2', place on magnetic rack for 5 min

11. Transfer 50 µL of sample to the first well of an 8-well strip tube

Right side size selection:

- 0.55 X ratio (µL of eluted sample from last step)
- 50 µL eluted sample X 0.55 = 27.5 µL of SPRISelect beads

1. Add 27.5 µL of SPRISelect beads to the well of the strip tube to which you have added the sample
2. Vortex for 1' (or pipet ten times), RT for 2'
3. Place strip tube on magnetic rack for 5' (there is a magnetic rack with wells in resembling a 96-well plate that you should be using for this)
4. Take 75uL of supernatant and transfer it to the next well in the strip tube
5. Concentrate the sample – 1.1 X ratio
    a. Add (50 µL + 27.5 µL) * 1.1 = 82.5 µL SPRISelect Beads into the second well
6. Vortex well for 1' (or pipet ten times), RT 2', place on magnetic rack for 5'
7. Remove supernatant
8. Wash sample with 180 µL of 85% ethanol (use P200 manual pipette), RT 1', X2
9. Use P12.5 electronic pipette to remove 10 µL from the bottom of the tube
10. Air dry RT 5', elute in 50 µL water and vortex well, RT 2', place on magnetic rack for 5'
11. Transfer 48 µL to a new tube and do bioanalysis (stopping point).

12. Check both the concentration and library size using Qubit and Tapestation, respectively.
13. The library is now read for final dilution and sequencing. Dilute as required by your designated sequencing facility.

5.5 Notes for Data Analysis

Primer sets should be trimmed from A and B libraries prior to merging the data from the same sample

a. ie. Trim A primers from A libraries and B primers from B libraries
b. After trimming, data can be merged to see full genome coverage