01. PURPOSE

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

*This protocol has been adapted from the ARTIC consortium protocol for nCoV-2019 sequencing which can be found here: https://artic.network/ncov-2019. Some primer sequences used in this protocol are from the following publication:

> *Gonzalez-Reiche, A., Hernandez, M., Sullivan, M., Ciferri, et al.* (2020).
> **Introductions and early spread of SARS-CoV-2 in the New York City area**
> Science https://dx.doi.org/10.1126/science.abc1917

2. IMPORTANT MANUALS/PROTOCOLS

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

3. REQUIREMENTS

3.1 MATERIALS

Amplification
One Step Ahead RT-PCR System (Qiagen: 220213)
RNase-free, DEPC-treated 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
Nextera DNA Library Prep Kit (Illumina: FC-121-10xx)
Nextera Index Kit (Illumina: FC-121-10xx)
IDT For Illumina DNA/RNA UD Indices (Illumina: 20027213)
25mL reagent reservoirs (e.g. VWR SR-0025-1SWM)
AMPure XP beads (Beckman Coulter A63880)
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)
Multichannel pipettes (P10, P20, P200, P1000)
Qubit fluorometer (Thermo Fisher)
Microplate centrifuge
TapeStation/Bioanalyzer (Agilent)
PCR plate roller/sealer (e.g. Bio-Rad MSR0001)

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 samples is:

Artic 1L: 5' – AACAAACCAACCAACTTTCGATCTC – 3'
Artic 6R: 5' – AACACGCACAGAATTTTGAGCAG – 3'
A2-F: 5' – TGGAACTTACACCAGTTGTTCAGAC – 3'
A2-R: 5' – AGCATCTTGTAGAGCAGGTGGA – 3'
A3-F: 5' – GCTACAATTGTGTACTTTTACTAGAA– 3'
A3-R: 5' – TCACGAGTGACACCACCATCAA – 3'
A4-F: 5' – ACGGTCTTTGGCTTGATGACGT– 3'

A4-R: 5' – TTTGACCGTGATGCAGCCATGC – 3'
A5-F: 5' – GCTAAATTCCTAAAAACTAATTGTTGTCGC – 3'
A5-R: 5' – GCGGACATACTTATCGGCAATTTTGTTA – 3'
A6-F: 5' – AACCTAGACCACCACTTAACCG – 3'
A6-R: 5' – CGCTTAACAAAGCACTCGTGGA – 3'
A7-F: 5' – GACTAGCTAAACGTTTTAAGGAATCACC – 3'
A7-R: 5' – TCCAACCTGAAGAAGAATCACC – 3'
A8-F: 5' – ACCCATTGGTGCAGGTATATGC – 3'
A8-R: 5' - TGCAGTAGCGCGAACAAAATCT – 3'
A9-F: 5' – TGTGGCTCAGCTACTTCATTGC – 3'
A9-R: 5' – GGCCCAGTTCCTAGGTAGTAGAAAT – 3'

The primer set for pool B samples is:

B1-F: 5' – GGTGCCTGGAATATTGGTGAACA – 3'
B1-R: 5' – GCCGACAACATGAAGACAGTGT – 3'
B2-F: 5' – GGTCCAACTTATTTGGATGGAGCTGAT – 3'
B2-R: 5' – AAAACACNTAAAGCAGCGGTTGA – 3'
B3-F: 5' – GTCACAACATTGCTTTGATATGGAACG – 3'
B3-R: 5' – TGGGCCTCATAGCACATTGGTA – 3'
B4-F: 5' – ATTGTGGGCTCAATGTGTCCAG – 3'
B4-R: 5' – AGCATAGACGAGGTCTGCCATT – 3'
B5-F: 5' – CCTAAATGTGATAGAGCCATGCCT – 3'
B5-R: 5' – TGCGAGCAGAAGGGTAGTAGAG – 3'
B6-F: 5' – CTGAGCGCACCTGTTGTCTATG – 3'
B6-R: 5' – TGAACCTGTTTGCGCATCTGTT – 3'
B7-F: 5' – TGGATGGAAAGTGAGTTCAGAG – 3'
B7-R: 5' – GCATTCAGTTGAATCACCACAAA – 3'
B8-F: 5' – GCTGTAGTTGTCTCAAGGGCTGTTGTT – 3'
B8-R: 5' - GCTCCCAATTTGTAATAAGAAAGC – 3'
B9-F: 5' – ACTTGTCACGCCTAAACGAACA – 3'
B9-R: 5' – TAGGCAGCTCTCCCTAGCATTG – 3'

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 of pool A primers in pool B and vice versa.

Primer pools A and B should contain all primers from each pool (9 sets, 18 primers) in total), pooled at equal volumes with the exception of primer sets A2, A3, and A4 in pool A and B7 in pool B, as listed below. Scale primer pools accordingly to accommodate all samples.

Primer (pool A)	Ratio in Pool	Primer (pool B)	Ratio in Pool
A2-F, A2-R	1.5x	B7-F, B7-R	1.25X
A3-F, A3-R	1.5x		
A4-F, A4-R	1.25x		

**All other primers are 1X

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.

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

1. Thaw RNA samples on ice. 10μM primers 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 as follows. Enzyme should be added last and kept on a cold block throughout the setup.

mRT-PCR – pool A1 reaction	**mRT-PCR – pool B1 reaction**
6μL DEPC-treated water	6μL DEPC-treated water
10μL 2.5X RT-PCR Master Mix	10μL 2.5X RT-PCR Master Mix
3μL 10μM primer pool A	3μL 10μM primer pool A
<u>1μL OneStep Ahead RT Mix</u>	<u>1μL OneStep Ahead RT Mix</u>
= 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. 68°C for 5'; 4°C hold

5. Run 2µL of each reaction on a 0.8% agarose gel to confirm M-RTPCR amplification. 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

The following is a modified version of the protocol detailed in Baym et al., 2015. Nextera chemistry is used to "tagment" (i.e. tag and fragment) the cDNA template, adding Illumina sequencing adapters directly to the amplicons from Step 4. Individual reactions are scaled volumetrically (1:4) in order to reduce substantially the cost of reagents per library.

Nextera chemistry's tagmentation solves each of these issues but requires highly accurate cDNA quantitation as input for library prep and may result in low coverage of the termini (~50nt) of each genome segment.

- Final libraries should be assessed on both the Qubit and Tapestation platforms to confirm successful construction and amplification.
- Libraries may be combined based on Qubit readings of concentration for a near-equimolar sequencing pool.

5.3.1 Initial M-RTPCR Amplicon Cleanup

The following protocol can be completed with the use of multichannel pipettes as described below but is best automated for e.g. the Agilent Bravo or other liquid handling platforms.

1. Bring the volume of each PCR reaction to 50µL with dH$_2$O
2. Allow AMPure XP beads to warm to room temperature before thoroughly resuspending beads by vortexing. During this time prepare a <u>fresh</u> volume of 80% ethanol for bead washing.
3. Add 0.6x beads by volume (i.e. 30µL) to each sample, vortexing as necessary to keep beads in solution. Pipette each sample thoroughly and incubate at room temperature for 5'.
4. Place samples on the appropriately sized magnetic rack until beads clump on the side of each tube/well, approximately 2-3 minutes. Remove and discard supernatant containing undesired small fragments.
5. Add 200µL 80% ethanol to wash each sample and incubate for 30s on the rack. Remove total volume of supernatant and discard. Don't resuspend the beads.
6. Repeat Step 5, this time carefully removing the full volume of wash solution first with a P200 pipette and next with a P10 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.
7. Working quickly, remove the samples from the rack and add 28µL RNase free, DEPC treated water to each well. Resuspend the entire bead mass by pipetting and incubate at room temperature for 5'.
8. Place samples back on the magnetic rack until beads clump and the supernatant is clear, approximately 3'. Remove 25µL 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 quantitation for input into the Nextera protocol:

1. Perform readings of samples using e.g. the Qubit dsDNA High Sensitivity assay with fresh standards. Sample readings should allow for consistent dilution to the required 0.2ng/µL for consistent Nextera tagmentation.
2. To ensure constant input volume and efficient tagementation of the purified, quantitated amplicons, calculate the volume of nuclease-free water required to dilute each sample to 0.2ng/µL. For samples with initial concentrations greater than 5ng/µL, serial dilution to 5ng/µL then 0.2ng/µL can be performed to improve

accuracy. Perform this dilution, pipetting or vortexing gently to mix and spinning down before use.

3. Confirm the concentration of several representative samples by Qubit dsDNA High Sensitivity assay.
4. Proceed to Nextera 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 Scaled Nextera Library Prep

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. The text is modified from an unpublished protocol shared by Sergey Kryazhimsky of the Desai Lab. Reaction volumes are approximately 1/4th that of the standard Nextera workflow and the protocol incorporates a reconditioning PCR step.

Tagmentation of cDNA
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 Tagment DNA Buffer (TD) and Amplicon Tagment Mix (ATM) reagents from the -20°C freezer and thaw on ice. After completely thawed, mix ATM by inverting and TD by gently vortexing. Spin down all reagents.
3. Make the Tagmentation Master Mix (TMM) by mixing *n* x 1.02 x 2.5μL TD and *n* x 1.02 x 1.25μl of ATM in a lo-bind tube. Mix thoroughly by gently pipetting the mixture up and down 20 times.
4. Distribute TMM into *r* PCR strip tubes with *c* x 1.02 x 3.75μL in each tube.
5. With a P10 multichannel pipette, distribute 3.75μL TMM into all appropriate wells of a fresh PCR plate (the "tagmentation plate").
6. With a P10 multichannel pipette, transfer 1.25μL of normalized 0.2ng/μL gDNA into the tagmentation plate for a total volume of 5μL per well. Mix gently by pipetting up and down 10 times with the same multichannel pipette, changing tips after every transfer.
(Nb: to speed up this process, one can simply pipette between the second stop and the rest position to mix the full 2.5μL reaction volume immediately after adding the normalized gDNA and without changing pipette tips.)
7. Cover plate with Microseal B, sealing tightly with a plate roller.
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 5'
   - Hold at 10°C

   (Nb: be sure that the thermal cycler lid is tightly sealed.)
10. During the incubation, remove the neutralize tagment buffer (NT) from the 4°C to bring to RT, and remove the NPM polymerase mix and the Nextera indices from the -20°C and thaw at room temperature.

    (Nb: it is crucial to avoid cross-contamination of the Nextera indices. Only open one at a time, use bleach to clean gloves or any spilled droplets)
11. Remove tagmentation plate from the thermal cycler as soon as the 5' at 55°C ends, spin briefly to collect condensation

    Neutralize Tagmentation
12. Immediately add 1.25µL NT to each well, pipetting to mix. Spin briefly and incubate for 5' at RT, then keep on ice or at 4°C until needed.

    Library Amplification/Barcoding
13. There are 2 indexing systems you can use: IDT UD Indices or Nextera SDI Index Kit

    IDT For Illumina DNA/RNA UD Indices
    a. Add 3.75µL NPM into each sample from the tagmentation plate.
    b. Spin down index plate and handle the index plate very carefully to avoid cross-contamination of indices. Add 2.5µL of unique index into each sample (when working with samples on plates, it works well to orient the tagmentation plate and plate of indices the same way then use the index from the index plate the corresponds to each well of the tagmentation plate) Change tips after every transfer.

    Nextera Index Kit
    c. Make r Row Master Mixes (RMMs), one for each row, by mixing c x 1.02 x 1.88µL of NPM and c x 1.02 x 1.25µL of each of the i5 indices in each of r PCR strip tubes. Make sure to order the indices meaningfully and label each tube with the index number. Mix thoroughly by pipetting the mixture up and down 20 times.
    d. Make c Column Master Mixes (CMMs), one for each column, by mixing r x 1.02 x 1.88µL NPM and r x 1.02 x 1.25µL of each of the i7 indices into c PCR strip tubes. Make sure to order the indices meaningfully and label each tube with the index number. Mix thoroughly by pipetting the mixture up and down 20 times.

| SARS-CoV2 Sequencing |
| Ghedin Lab |
| *Standard Operating Procedure* |

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     e. Arrange the CMMs just above or below the tagmentation plate, using a plate holder to stabilize the strip tube. (Nb: a 10uL pipette tip insert wafer works well for this purpose.)

     f. Using a P10 multichannel, transfer 3.1µL of CMM into each reaction well such that each column receives the same i7 index. Change tips after every transfer.

     g. Arrange the RMMs to the left or right of the tagmentation plate. Using a P10 multichannel, transfer 3.1µL of RMM into each reaction well, such that each column receives the same i5 index. Change tips after every transfer. Mix each reaction gently by pipetting up and down 10 times (final total volume per well = 12.5µL).

14. Cover plate, making sure to press firmly with a plate roller on each well, especially those around the edges.
15. Spin plate briefly to collect reaction volume at the bottom of each well (e.g. 1000g for 1').
16. Place the plate in a pre-heated thermal cycler and run the following program with a heated lid:
    - 72°C for 3'
    - 95°C for 30s
    - [95°C for 10s; 55°C for 30s; 72°C for 30s] x 12 cycles
    - 72°C for 5'
    - 4°C hold
17. When complete, remove plate from the thermal cycler, spin briefly to collect condensation, and place on ice until needed.
    Dilute, Clean and Pool Libraries
18. Bring volume or the reactions to 50µL with dH_2O
19. Clean libraries with a 0.9X bead clean-up. Follow steps in Section 5.3.1 using 0.9X volume of beads (45µL to a 50µL reaction volume). Following drying, elute in 28µL of dH_2O
20. Perform readings of samples using the Qubit dsDNA High Sensitivity assay with fresh standards to determine post-amplification concentration.
21. Use Tapestation (D1000, HS) to check library quality and fragment size (2µL sample or ladder, 2µL buffer).
22. If both size and concentrations look sufficient, proceed to pooling of libraries
23. 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 in order to calculate individual molarities for each library. Determine the minimum concentration needed in order to pool the samples equimolarly.

24. Calculate and add the appropriate amount of DEPC treated water to samples in order to dilute to the minimum concentration. Mix well by pipeting.
25. Combine equal volumes of each sample into one tube, pipet to mix.
26. Run pooled libraries on high sensitivity D1000 Tapestation to look at fragment size distribution.
27. Perform a 0.55x bead clean-up of the pooled library using Ampure beads (refer to section 5.3.1 for bead clean-up protocol, using a 0.55x bead to sample ratio in Step 3).
28. Following the elution step, check both the concentration and library size using Qubit and Tapestation, respectively.
29. The library is now read for final dilution and sequencing. Dilute as required by your designated sequencing facility.

5.4 Notes for Data Analysis

1. 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