

SARS-CoV2 Sequencing Ghedin Lab Standard Operating Procedure		
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AUTHOR: <i>Allison Roder</i>		PRIMARY REVIEWER:

## 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

Superscript III ([Thermo Fisher](#)) or SuperScript IV One-Step RT-PCR System ([Thermo Fisher](#))  
Nextera DNA Library Prep kit (Illumina) & Indices

## 3. REQUIREMENTS

### 3.1 MATERIALS

#### **Amplification**

SuperScript III One-Step RT-PCR System (Thermo Fisher: 12574018) or  
SuperScript IV One-step RT-PCR System (ThermoFisher: 12594100)  
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)  
TE Buffer (10mM Tris, 0.1mM EDTA, pH 8.0)

#### **Library Prep**

Nextera DNA Library Prep Kit (Illumina: FC-121-10xx)  
Nextera Index Kit (Illumina: FC-121-10xx)  
KAPA Library Amplification Kit (KAPA Biosystems: KK2611/2612)

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25mL reagent reservoirs (e.g. VWR SR-0025-1SWM)  
 AMPure XP beads (Beckman Coulter A63880)  
 Qubit dsDNA HS Assay Kit (Thermo Fisher: Q32854)  
 Quant-iT DNA Assay Kit, high sensitivity (Thermo Fisher: Q-33120)  
 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)  
 Qubit fluorometer (Thermo Fisher)  
 Microplate reader (e.g. Tecan Infinite 200 Pro)  
 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:

A1-F: 5' – CCAGGTAACAAACCAACCAACTTT – 3'  
 A1-R: 5' – GCCACTGCGAAGTCAACTGAACA – 3'

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A2-F:	5' – TGGAACCTTACACCAGTTGTTTCAGAC – 3'
A2-R:	5' – AGCATCTTGTAGAGCAGGTGGA – 3'
A3-F:	5' – AAACCGTGTTTGTACTAATTATATGCCTT – 3'
A3-R:	5' – TCACGAGTGACACCACCATCAA – 3'
A4-F:	5' – ACGGTCTTTGGCTTGATGACGT – 3'
A4-R:	5' – GCATGGCTGCATCACGGTCAAA – 3'
A5-F:	5' – GCTAAATTCCTAAAACTAATTGTTGTCGC – 3'
A5-R:	5' – GCGGACATACTTATCGGCAATTTTGTTA – 3'
A6-F:	5' – TGTTGGTGATTATTTTGTGCTGACAT – 3'
A6-R:	5' – CGCTTAACAAAGCACTCGTGGA – 3'
A7-F:	5' – ACCCAGGAGTCAAATGGAAATTGA – 3'
A7-R:	5' – CCTGAGGGAGATCACGCACTAA – 3'
A8-F:	5' – ACCCATTTGGTGCAGGTATATGC – 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' – GGTCCAACCTATTTGGATGGAGCTGAT – 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' – TTCGAAGACCCAGTCCCTACTT – 3'
B7-R:	5' – AGTGACACTTGCAGATGCTGGCT – 3'
B8-F:	5' – GCTGTAGTTGTCTCAAGGGCTGTTGTT – 3'
B8-R:	5' – GCTCCCAATTTGTAATAAGAAAGC – 3'
B9-F:	5' – ACTTGTACGCCTAAACGAACA – 3'
B9-R:	5' – TAGGCAGCTCTCCCTAGCATTG – 3'

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## Sequencing

- We have routinely achieved sufficient coverage for ~70-80 reactions (35-40 samples) on the MiSeq platform using V3 chemistry for 2x300 paired-end reads. We anticipate that up to 50 samples can be pooled under these conditions if sufficient library barcodes are available.

## 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

- Thaw RNA samples on ice. 10µM primers and SSIII 2X reaction mix (excluding enzyme) may be thawed at room temperature, vortexed, and placed on ice.
- 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**  
2.5µL DEPC-treated water

**mRT-PCR – pool B1 reaction**  
2.5µL DEPC-treated water

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12.5µL 2X RT-PCR buffer  
3µL 10µM primer pool A  
1µL DMSO  
0.5µL SSIII One-step enzyme  
= 20µL total

12.5µL 2X RT-PCR buffer  
3µL 10µM primer pool B  
1µL DMSO  
0.5µL SSIII RT One-step enzyme  
= 20µL total

[Note: SSIV One-Step has also been tested with this protocol. To use SSIV, decrease enzyme to 0.25µL and adjust water to keep mastermix to 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 42°C with a heated lid offset of 10°C.
4. 4-stage cycling parameters:
  - a. 45°C for 60'; 94°C for 2'
  - b. [94°C for 15s; 55°C for 30s; 68°C for 8'] x 12 cycles\*
  - c. [98°C for 15s; 55°C for 30s; 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-RT-PCR 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.

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- 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

**\*\*If amplicons look relatively clear of non-specific bands on gel, skip this step\*\***

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 TE buffer.
2. Allow AMPure XP beads to warm to room temperature before thoroughly resuspending beads by vortexing. During this time prepare a fresh volume of 80% ethanol for bead washing.
3. Add 1.8x beads by volume (i.e. 90µL) 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 7, 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 5-10' 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 TE buffer 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 5'. Remove 25µL to a clean, labeled LoBind tube or PCR plate for storage, minimizing bead carryover.

### 5.3.2 Quantitation and Dilution

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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 Quant-iT DNA Assay kit (High Sensitivity) with fresh standards.
2. To ensure constant input volume and efficient tagmentation of the purified, quantitated amplicons, calculate the volume of nuclease-free water required to dilute each sample to 0.2ng/μL. 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 1/4<sup>th</sup> 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. Once completely thawed, mix ATM by inverting and TD by gently vortexing. Spin down all reagents.
3. Make the Tagmentation Master Mix (TMM) by mixing  $n \times 1.02 \times 2.5\mu\text{L}$  TD and  $n \times 1.02 \times 1.25\mu\text{L}$  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  $c \times 1.02 \times 3.75\mu\text{L}$  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 gDNA into the tagmentation plate for a total volume of 5μL per well. Mix gently by pipetting up

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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 6'
  - 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. Mix the KAPA reagent by inverting and the indices by vortexing gently before spinning down.  
(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, and if possible prepare single-use aliquots in PCR strip tubes.)
11. Remove tagmentation plate from the thermal cycler, spin briefly to collect condensation, and place on ice until needed.

#### **Neutralize Tagmentation**

12. Add 1.25µL NT to each well, pipetting to mix. Spin briefly and incubate for 5' at RT.

#### **Library Amplification/Barcoding**

13. Make  $r$  Row Master Mixes (RMMs), one for each row, by mixing  $c \times 1.02 \times 1.88\mu\text{L}$  of NPM and  $c \times 1.02 \times 1.25\mu\text{L}$  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.
14. Make  $c$  Column Master Mixes (CMMs), one for each column, by mixing  $r \times 1.02 \times 1.88\mu\text{L}$  NPM and  $r \times 1.02 \times 1.25\mu\text{L}$  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.
15. 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.)



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- 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.
16. 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).
  17. Cover plate, making sure to press firmly with a plate roller on each well, especially those around the edges.
  18. Spin plate briefly to collect reaction volume at the bottom of each well (e.g. 1000g for 1').
  19. 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
  20. When complete, remove plate from the thermal cycler, spin briefly to collect condensation, and place on ice until needed.
- Dilute, Clean and Pool Libraries**
21. Bring volume of the reactions to 50µL with dH<sub>2</sub>O
  22. Clean libraries with a 0.9X bead clean-up. Follow steps in Section 5.3.1 using 0.9X volume of beads (40µL to a 50µL reaction volume). Following drying, elute in 20µL of dH<sub>2</sub>O
  23. Perform readings of samples using the Quant-iT DNA Assay kit (High Sensitivity) with fresh standards to determine post-amplification concentration.
  24. Use Tapestation (D1000, HS) to check library quality and fragment size (2µL sample or ladder, 2µL buffer).
  25. If both size and concentrations look sufficient, proceed to pooling of libraries
  26. Libraries should be pooled at equal molarity. Input both the average fragment size of the library and the concentration as calculated by Qubit into the [KAPA Dilution Determining calculator](#) in order to calculate individual molarities for each library. Determine the minimum concentration needed in order to pool the samples.
  27. Calculate and add the appropriate amount of DEPC treated water to samples in order to dilute to the minimum concentration. Mix well by pipeting.

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28. Briefly spin down the plate and check a representative number of samples using the Qubit to ensure they were accurately diluted to the intended molarity.
29. Combine equal volumes of each sample into one tube, pipet to mix.
30. Perform a 0.7x bead clean-up of the pooled library using Ampure beads (refer to section 5.3.1 for bead clean-up protocol, using a 0.7x bead to sample ratio (32.5  $\mu$ L beads:50 $\mu$ L sample) in Step 3).
31. Following the elution step, check both the concentration and library size using Qubit and TapeStation, respectively.
32. The library is now ready 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