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SARS-CoV-2 McGill Nextera Flex sequencing protocol_Lunascript_ARTIC.V3_5uLRT

 [SARS-CoV-2 McGill Nextera Flex sequencing protocol_SS_V3_LA1_5uLRT](#)

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How the Nextera DNA Flex Assay Works

The Nextera DNA Flex library prep kit uses a bead-based transposome complex to tagment genomic DNA, which is a process that fragments DNA and then tags the DNA with adapter sequences in one step. After it is saturated with input DNA, the bead-based transposome complex fragments a set number of DNA molecules. This fragmentation provides flexibility to use a wide DNA input range to generate normalized libraries of consistent tight fragment size distribution. Following tagmentation, a limited-cycle PCR adds Nextera DNA Flex-specific index adapter sequences to the ends of a DNA fragment. This step enables capability across all Illumina sequencing platforms. A subsequent Sample Purification Beads (SPB) cleanup step then purifies libraries for use on an Illumina sequencer. The double-stranded DNA library is denatured before hybridization of the biotin probe oligonucleotide pool.

PCR Amplicons for Nextera Flex

When starting with PCR amplicons, the PCR amplicon must be > 150 bp. The standard clean up protocol depletes libraries < 500 bp. **Therefore, Illumina recommends that amplicons < 500 bp undergo a 1.8 x sample purification bead volume ratio to supernatant during Clean Up Libraries on page 11.** Shorter amplicons can otherwise be lost during the library cleanup step. Tagmentation cannot add an adapter directly to the distal end of a fragment, so a drop in sequencing coverage of ~50 bp from each distal end is expected. To ensure sufficient coverage of the amplicon target region, design primers to extend beyond the target region by 50 bp per end.

More information can be found here:

https://emea.support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/samplepreps_nextera/nextera_dna_flex/nextera-dna-flex-library-prep-reference-guide-1000000025416-07.pdf

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cDNA preparation

1 cDNA PREPARATION

Mix the following components in a 0.2 mL 8-strip tube or in a 96-well plate:

Component	Volume
LunaScript RT SuperMix (5x)	4 µL
Nuclease-free water	5 µL
Template RNA	11 µL
Total	20 µL

Viral RNA input from a clinical sample should be between Ct 18-35. If Ct is between 12-15, then dilute the sample 100-fold in water, if between 15-18 then dilute 10-fold in water. This will reduce the likelihood of PCR-inhibition.

A mastermix should be made up in the **mastermix cabinet** and aliquoted into PCR strip tubes. Tubes should be wiped down when entering and leaving the mastermix cabinet.

2 Gently mix by pipetting and pulse spin the tube to collect liquid at the bottom of the tube.

3 Incubate the reaction as follows:

24m

25 °C for 00:02:00

⌚ 55 °C for ⌚ 00:20:00

⌚ 95 °C for ⌚ 00:01:00

Place on ice for ⌚ 00:01:00 or store cDNA at -20C





Primer pool preparation

4 PRIMER POOL PREPARATION

If required resuspend lyophilised primers at a concentration of 100 µM each

- 5 ARTIC nCov-2019 only primers for this protocol were designed using Primal Scheme and generate overlapping 400 nt amplicons. Primer names and dilutions are listed in the table below. https://github.com/sarahreiling/artic-ncov2019/blob/master/primer_schemes/nCoV-2019/V3/nCoV-2019_V3only.scheme.bed

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Generate primer pool stocks by adding  5 µL of each primer pair to a  1.5 mL Eppendorf labelled either “Pool 1 (100 µM)” or “Pool 2 (100 µM)”. Total volume should be  490 µL for Pool 1 (100 µM) and  490 µL for Pool 2 (100 µM). These are your 100 µM stocks of each primer pool.

Primers should be diluted and pooled in the **mastermix cabinet** which should be cleaned with decontamination wipes and UV sterilised before and after use.

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Dilute this primer pool 1:10 in molecular grade water, to generate 10 µM primer stocks. It is recommend that multiple aliquots of each primer pool are made to in case of degradation or contamination.

Primers need to be used at a final concentration of 0.015 µM per primer. In this case both pools have 98 primers in so the requirement is 3.65 µL primer pools (10 uM) per 25 µL reaction. For other schemes, adjust the volume added appropriately.

Multiplex PCR

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MULTIPLEX PCR

In the **extraction and sample addition cabinet** add **5 µL** RT product to each tube and mix well by pipetting.

The **extraction and sample addition cabinet** should be cleaned with decontamination wipes and UV sterilised before and after use.

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In the mastermix hood set up the multiplex PCR reactions as follows in 0.2mL 8-strip PCR tubes:

Component µM]	Pool 1 [10 uM primer]	Pool 2 [10
Q5 Hot Start High-Fidelity 2X Master Mix	12.5 µL	12.5 µL
Primer Pool 1 or 2	3.7 µL	3.7 µL
Nuclease-free water	3.8 µL	3.8 µL
Total	20 µL	20 µL

Add 5 ul RT product as mentioned in step 10.

A PCR mastermix for each pool should be made up in the **mastermix cabinet** and aliquoted into PCR strip tubes. Tubes should be wiped down when entering and leaving the mastermix cabinet.

10 Pulse centrifuge the tubes to collect the contents at the bottom of the tube.

11 Set-up the following program on the thermal cycler:

Step	Temperature	Time	Cycles
------	-------------	------	--------

Heat Activation	98 °C	00:00:30	1
Denaturation	98 °C	00:00:15	36
Annealing	63 °C	00:05:00	36
Hold	4 °C	Indefinite	1

Cycle number should be 25 for Ct 18-21 up to a maximum of 36 cycles for Ct 35

PCR clean-up

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PCR CLEANUP

Combine the entire contents of "Pool 1" and "Pool 2" PCR reactions for each biological sample into to a single 1.5 mL Eppendorf tube.

13 Clean-up the amplicons using the following protocol:

Add a volume ratio of 0.8x of SPRI beads to the sample tube and mix gently by either flicking or pipetting.

Incubate for 5 min at room temperature.

Pellet on magnet for 5 min. Remove supernatant.

Add 200 ul of 80% ethanol to the pellet and wash twice.

Elute in 30 ul elution buffer.

Amplicon clean-up should be performed in the **post-PCR cabinet which** should should be cleaned with decontamination wipes and UV sterilised before and after use.

Amplicon Quantification and normalisation

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AMPLICON QUANTIFICATION AND NORMALIZATION

Quantify the amplicon pools using a fluorimetric dsDNA assay.

We expect following concentrations:

Pool 1+2 combined:

100-150 ng/ul for Ct 14-24

30-80 ng/ul for Ct 25-29

10-30 ng/ul for Ct 30-36

- 15 Use the 30 ul with 150 ng DNA for Nextera Flex Tagmentation.

Protocol can be found here:

https://emea.support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/samplepreps_nextera/nextera_dna_flex/nextera-dna-flex-library-prep-reference-guide-1000000025416-07.pdf

Start on page 7 with the Tagmentation.

Tagment Genomic DNA

16 Tagment Genomic DNA

This step uses the Bead-Linked Transposomes (BLT) to tagment DNA, which is a process that fragments and tags the DNA with adapter sequences.

Consumables

- ▶ Bead-Linked Transposomes (BLT)
- ▶ Tagmentation Buffer 1 (TB1)
- ▶ Nuclease-free water
- ▶ 96-well PCR plate
- ▶ Microseal 'B' adhesive seal
- ▶ 1.7 ml microcentrifuge tubes
- ▶ 8-tube strip
- ▶ Pipette tips
 - ▶ 20 µl multichannel pipettes
 - ▶ 200 µl multichannel pipettes

About Reagents

- ▶ BLT must be stored at temperatures above 2°C. Do not use BLT that has been stored below 2°C.

Preparation

- 1 Prepare the following consumables:

Item	Storage	Instructions
BLT	2°C to 8°C	Bring to room temperature. Vortex to mix. Do not centrifuge before pipetting.
TB1	-25°C to -15°C	Bring to room temperature. Vortex to mix.

- 2 Save the following TAG program on the thermal cycler:

- ▶ Choose the preheat lid option and set to 100°C
- ▶ Set the reaction volume to 50 µl
- ▶ 55°C for 15 minutes
- ▶ Hold at 10°C

Procedure

- 1 Add 2–30 µl DNA to each well of a 96-well PCR plate so that the total input amount is 100–500 ng.
- 2 If DNA volume < 30 µl, add nuclease-free water to the DNA samples to bring the total volume to 30 µl.
- 3 Vortex BLT (yellow cap) vigorously for 10 seconds to resuspend. Repeat as necessary.
- 4 Combine the following volumes to prepare the tagmentation master mix. Multiply each volume by the number of samples being processed.
 - ▶ BLT (11 µl)
 - ▶ TB1 (11 µl)Reagent overage is included in the volume to ensure accurate pipetting.
- 5 Vortex the tagmentation master mix thoroughly to resuspend.
- 6 Divide the tagmentation master mix volume equally into an 8-tube strip.
- 7 Using a 200 µl multichannel pipette, transfer 20 µl tagmentation master mix to each well of the plate containing a sample. Use fresh tips for each sample column.
- 8 Discard the 8-tube strip after the tagmentation master mix has been dispensed.
- 9 Pipette each sample 10 times to resuspend. Use fresh tips for each sample column.
- 10 Seal the plate with Microseal 'B', place on the preprogrammed thermal cycler, and run the TAG program.

For automated protocol : at step 7, 22 ul tagmentation master mix is transferred.

Post Tagmentation Cleanup

17 Post Tagmentation Cleanup

This step washes the adapter-tagged DNA on the BLT before PCR amplification.

Consumables

- ▶ Tagment Stop Buffer (TSB)
- ▶ Tagment Wash Buffer (TWB)
- ▶ 96-well plate magnet
- ▶ Microseal 'B' adhesive seal
- ▶ Pipette tips
 - ▶ 20 µl multichannel pipettes
 - ▶ 200 µl multichannel pipettes

Preparation

- 1 Prepare the following consumables:

Item	Storage	Instructions
TSB	15°C to 30°C	If precipitates are observed, heat at 37°C for 10 minutes, and then vortex until precipitates are dissolved. Use at room temperature.
TWB	15°C to 30°C	Use at room temperature.

- 2 Save the following PTC program on the thermal cycler:

- ▶ Choose the preheat lid option and set to 100°C
- ▶ Set the reaction volume to 60 µl
- ▶ 37°C for 15 minutes
- ▶ Hold at 10°C

Procedure

- 1 Add 10 µl TSB to the tagmentation reaction.
- 2 Slowly pipette each well 10 times to resuspend the beads.
- 3 Seal the plate with Microseal 'B', place on the preprogrammed thermal cycler, and run the PTC program.
- 4 Place the plate on the magnetic stand and wait until liquid is clear (~3 minutes).
- 5 Using a multichannel pipette, remove and discard supernatant.

- 6 Wash two times as follows:
 - a Remove the sample plate from the magnetic stand and use a deliberately slow pipetting technique to add 100 µl TWB directly onto the beads. A deliberately slow pipetting technique minimizes the potential of TWB foaming to avoid incorrect volume aspiration and incomplete mixing.
 - b Pipette slowly until beads are fully resuspended.
 - c Place the plate on the magnetic stand and wait until the liquid is clear (~3 minutes).
 - d Using a multichannel pipette, remove and discard supernatant.
- 7 Remove the plate from the magnetic stand and use a deliberately slow pipetting technique to add 100 µl TWB directly onto the beads.
- 8 Pipette each well slowly to resuspend the beads.
- 9 Seal the plate and place on the magnetic stand until the liquid is clear (~3 minutes). Keep on the magnetic stand until step 4 of the *Procedure* section in *Amplify Tagmented DNA*. The TWB remains in the wells to prevent overdrying of the beads.

Amplify Tagmented DNA

18 Amplify Tagmented DNA

This step amplifies the tagmented DNA using a limited-cycle PCR program. The PCR step adds Index 1 (i7) adapters, Index 2 (i5) adapters, and sequences required for sequencing cluster generation. To confirm the indexes selected for low plexity pooling have the appropriate color balance, see the *Index Adapters Pooling Guide* (document # 1000000041074).

Index adapter tubes or plates are ordered separately from the library prep components. For a list of compatible index adapters for use with this protocol, see *Kit Contents* on page 24.

Consumables

- ▶ Enhanced PCR Mix (EPM)
- ▶ Index adapters (tubes or plate)
- ▶ Nuclease-free water
- ▶ Microseal 'B' adhesive seal
- ▶ 1.7 ml microcentrifuge tubes
- ▶ Pipette tips
 - ▶ 20 µl multichannel pipettes
 - ▶ 200 µl multichannel pipettes

About Reagents

- ▶ Index adapter plates
 - ▶ A well may contain >10 µl of index adapters.
 - ▶ Do not add samples to the index adapter plate.
 - ▶ Each well of the index plate is single use only.
- ▶ Index adapter tubes
 - ▶ Open only one index adapter tube at a time to prevent misplacing caps. Alternatively, use fresh caps after opening each tube.

Preparation

- 1 Prepare the following consumables:

Item	Storage	Instructions
EPM	-25°C to -15°C	Thaw on ice. Invert to mix, then briefly centrifuge.
Index Adapters	-25°C to -15°C	Thaw at room temperature. [Tubes] Vortex to mix, then centrifuge briefly. [Plates] Spin briefly before use.

- 2 Save the following BLT PCR program on a thermal cycler using the appropriate number of PCR cycles, indicated in the table below.

- ▶ Choose the preheat lid option and set to 100°C
- ▶ 68°C for 3 minutes
- ▶ 98°C for 3 minutes
- ▶ (X) cycles of:
 - ▶ 98°C for 45 seconds
 - ▶ 62°C for 30 seconds
 - ▶ 68°C for 2 minutes
- ▶ 68°C for 1 minutes
- ▶ Hold at 10°C

Total DNA Input (ng)	Number of PCR Cycles (X)
1–9	12
10–24	8
25–49	6
50–99	5
100–500	5
Blood/Saliva	5

Procedure

- 1 Combine the following volumes to prepare the PCR master mix. Multiply each volume by the number of samples being processed.
 - ▶ EPM (22 µl)
 - ▶ Nuclease-free water (22 µl)Reagent overage is included in the volume to ensure accurate pipetting.
- 2 Vortex, and then centrifuge the PCR master mix at 280 × g for 10 seconds.
- 3 With the plate on the magnetic stand, use a 200 µl multichannel pipette to remove and discard supernatant.
Foam that remains on the well walls does not adversely affect the library.
- 4 Remove from the magnet.
- 5 Immediately add 40 µl PCR master mix directly onto the beads in each sample well.
- 6 Immediately pipette to mix until the beads are fully resuspended. Alternatively, seal the plate and use a plate shaker at 1600 rpm for 1 minute.
- 7 Seal the sample plate and centrifuge at 280 × g for 3 seconds.
- 8 Add the appropriate index adapters to each sample.

Index Kit Type	Kit Configuration	Volume of Index Adapter per Sample
24 plex (dual index)	Individual tubes	5 µl i7 adapter 5 µl i5 adapter
96 plex (dual index)	96-well plate	10 µl pre-paired i7 and i5 index adapters

- 9 Using a pipette set to 40 µl, pipette 10 times to mix. Alternatively, seal the plate and use a plate shaker at 1600 rpm for 1 minute.
- 10 Seal the plate with Microseal 'B', and then centrifuge at 280 × g for 30 seconds.
- 11 Place on the thermal cycler and run the BLT PCR program.

SAFE STOPPING POINT

If you are stopping, store at 2°C to 8°C for up to 3 days.

For automated protocol : at step 9, 44 ul tagmentation master mix is transfered.

19 Clean Up Libraries

This step uses double-sided bead purification procedure to purify the amplified libraries.

Consumables

- ▶ Sample Purification Beads (SPB)
- ▶ Resuspension Buffer (RSB)
- ▶ Freshly prepared 80% ethanol (EtOH)
- ▶ 96-well 0.8 ml Polypropylene Deepwell Storage Plate (midi plate) (2)
- ▶ 96-well PCR plate
- ▶ Microseal 'B' adhesive seal
- ▶ Microseal 'F' foil seal
- ▶ 1.7 ml microcentrifuge tubes
- ▶ Nuclease-free water

About Reagents

- ▶ Sample Purification Beads
 - ▶ Must be at room temperature before use
 - ▶ Vortex before each use
 - ▶ Vortex frequently to make sure that beads are evenly distributed
 - ▶ Aspirate and dispense slowly due to the viscosity of the solution

Preparation

- 1 Prepare the following consumables:

Item	Storage	Instructions
SPB	2°C to 8°C	Let stand at room temperature for 30 minutes. Vortex and invert to mix.
RSB	-25°C to -15°C	Thaw and bring to room temperature. Vortex to mix.

- 2 Prepare fresh 80% EtOH from absolute ethanol.

Procedure

- 1 Centrifuge at $280 \times g$ for 1 minute to collect contents at the bottom of the well.
- 2 Place the plate on the magnetic stand and wait until the liquid is clear (~5 minutes).
- 3 Transfer 45 μ l supernatant from each well of the PCR plate to the corresponding well of a new midi plate.
- 4 Vortex and invert SPB multiple times to resuspend.
- 5 For standard DNA input, perform the following steps.
 - a Add 40 μ l nuclease-free water to each well containing supernatant.
 - b Add 45 μ l SPB to each well containing supernatant.
 - c Pipette each well 10 times to mix. Alternatively, seal the plate and use a plate shaker at 1600 rpm for 1 minute.
 - d Seal the plate and incubate at room temperature for 5 minutes.
 - e Place on the magnetic stand and wait until the liquid is clear (~5 minutes).
 - f During incubation, thoroughly vortex the SPB (*undiluted* stock tube), and then add 15 μ l to each well of a *new* midi plate.
 - g Transfer 125 μ l supernatant from each well of the first plate into the corresponding well of the second plate (containing 15 μ l *undiluted* SPB).
 - h Pipette each well in the second plate 10 times to mix. Alternatively, seal the plate and use a plate shaker at 1600 rpm for 1 minute.
 - i Discard the first plate.
- 6 For small PCR amplicon input, perform the following steps.
 - a Add 81 μ l SPB to each midi plate well containing supernatant.
 - b Pipette each well 10 times to mix. Alternatively, seal the plate and use a plate shaker at 1600 rpm for 1 minute.
- 7 Incubate the sealed midi plate at room temperature for 5 minutes.
- 8 Place on the magnetic stand and wait until the liquid is clear (~5 minutes).
- 9 Without disturbing the beads, remove and discard supernatant.
- 10 Wash two times as follows.
 - a With the plate on the magnetic stand, add 200 μ l fresh 80% EtOH without mixing.
 - b Incubate for 30 seconds.
 - c Without disturbing the beads, remove and discard supernatant.
- 11 Use a 20 μ l pipette to remove and discard residual EtOH.
- 12 Air-dry on the magnetic stand for 5 minutes.
- 13 Remove from the magnetic stand.
- 14 Add 32 μ l RSB to the beads.
- 15 Pipette to resuspend.
- 16 Incubate at room temperature for 2 minutes.
- 17 Place the plate on the magnetic stand and wait until the liquid is clear (~2 minutes).
- 18 Transfer 30 μ l supernatant to a new 96-well PCR plate.

SAFE STOPPING POINT

If you are stopping, seal the plate with Microseal 'B' adhesive or Microseal 'F' foil seal, and store at -25°C to -15°C for up to 30 days.

At step 14, beads can be resuspended in 62 μ L RSB, followed by a 60 μ L transfer of supernatant at step 18. This is used to decrease the concentration of the final pool in order to facilitate the QC step.

Pool Libraries

20 Pool Libraries

When the DNA input is 100-500 ng, quantifying and normalizing individual libraries generated in the same experiment is not necessary. However, the final yield of libraries generated in separate experiments can vary slightly.

To achieve optimal cluster density, pool equal library volumes and quantify the pool before sequencing.

DNA Inputs of 100–500 ng

- 1 Combine 5 μ L of each library (up to 384 libraries) in a 1.7 ml microcentrifuge tube.
- 2 Vortex to mix, and then centrifuge.
- 3 Quantify the library pool using a dsDNA fluorescent dye method, such as Qubit or PicoGreen.

For DNA Inputs of < 100 ng

- 1 Quantify each library individually using Qubit or PicoGreen.

Check Library Quality

21 Check Library Quality (Optional)

- 1 Run 1 μ L library or pooled libraries on one of the following instruments:
 - ▶ Advanced Analytical Fragment Analyzer with the HS-NGS High Sensitivity 474 kit.
 - ▶ Add 1 μ L RSB to the library to achieve the 2 μ L volume required for Fragment Analyzer.
 - ▶ Agilent 2100 Bioanalyzer with a High Sensitivity DNA kit.

Dilute Libraries to the Starting Concentration

Dilute Libraries to the Starting Concentration

This step dilutes libraries to the starting concentration for your sequencing system and is the first step in a serial dilution. After diluting to the starting concentration, libraries are ready to be denatured and diluted to the final loading concentration.

For sequencing, Illumina recommends the read lengths indicated in the table below.

Table 2 Recommended Read Length on Illumina Systems

Sequencing System	Read Length
NovaSeq 6000, HiSeq X*, HiSeq 3000 and HiSeq 4000, NextSeq 500 and NextSeq 550, MiSeq, MiniSeq, iSeq 100	2 x 151
HiSeq 2000, HiSeq 2500 (high output)	2 x 126
HiSeq 2500 (rapid run)	2 x 101**

*Not compatible with IDT for Illumina Nextera DNA UD Indexes (10bp)

**Assumes the use of the 200 cycle kit

IDT for Illumina Nextera DNA UD Indexes uses 10 base pair index codes that differ from the Nextera DNA CD Indexes, and which use 8 base pair index codes. This change in base pair index codes can require adjustments to your sequencing run set up.

- Calculate the molarity value of the library or pooled libraries using the following formula.
 - For libraries qualified on a Bioanalyzer, use the average size obtained for the library.
 - For all other qualification methods, use 600 bp as the average library size.
$$\frac{\text{ng} / \mu\text{l} \times 10^6}{660 \frac{\text{g}}{\text{mol}} \times \text{average library size (bp)}} = \text{Molarity (nM)}$$
- Using the molarity value, calculate the volumes of RSB and library needed to dilute libraries to the starting concentration for your system.

Sequencing System	Starting Concentration (nM)	Final Loading Concentration (pM)
HiSeq 2500 and HiSeq 2000 (high output modes)	2	12
HiSeq 2500 (rapid run mode)	2	8.5
HiSeq X, HiSeq 4000, and HiSeq 3000	2–3	200–300
iSeq 100	2	200
MiniSeq	2	1.2–1.3
MiSeq (v3 reagents)	4	12
NextSeq 550 and NextSeq 500	2	1.2–1.3
NovaSeq 6000	2	See document # 1000000019358 (NovaSeq 6000 System Guide)

- Dilute libraries using RSB:
 - Libraries quantified as a multiplexed library pool**—Dilute the pool to the starting concentration for your system.
 - Libraries quantified individually**—Dilute each library to the starting concentration for your system. Add 10 µl each diluted library to a tube to create a multiplexed library pool.
- Follow the denature and dilute instructions for your system to dilute to the final loading concentration.
 - For the iSeq 100 System, see the system guide for dilution instructions (libraries are automatically denatured).
 - For the NovaSeq 6000 System, see the system guide for pool and denature instructions.
 - For the HiSeq 4000 and HiSeq 3000 Systems, see the cBot 2 or cBot system guide for reagent preparation instructions.
 - For all other systems, see the denature and dilute libraries guide.

The final loading concentrations are a starting point and general guideline. Optimize concentrations for your workflow and quantification method over subsequent sequencing runs or by flow cell titration.