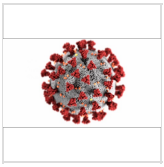

Version 1

Apr 29, 2020

SARS-CoV-2 Sequencing on Illumina MiSeq Using ARTIC Protocol: Part 2 - Illumina DNA Flex Protocol V.1

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Coronavirus Method Development Community StaPH-B view 1 more group

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ABSTRACT

This protocol is an adaption of several circulating protocols on SARS-CoV-2 sequencing using the ARTIC protocol and the Illumina Nexterra DNA Flex library prep kit. Its purpose is to simplify things for the average state public health laboratory, using equipment and expertise they currently posess, most likely from their funded PulseNet activities.

Feedback and comments appreciated.

STEPS MATERIALS

NAME	CATALOG #	VENDOR
Nextera DNA Flex Library Prep	20018705	Illumina, Inc.

DNA Flex - Before you begin

1

Before You Begin

This protocol is an adaptation of the **Illumina Nextera DNA Flex Library Prep** kit (below). It is laid out specifically for using amplicons from the ARTIC Protocol. The audience is assumed to be public health laboratorians with access to instruments and reagents used for PulseNet WGS.

Four documents are linked below for reference, and can also be found following the reagent link for the Nextera DENA Flex Library Prep. The consumables and equipment document (CED) shoud be used to make sure you have the appropriate materials before beginning. There are too many consumables to list out individually in this protocol so please use the document and fill out your Lot# and Exp Dates on this document as well. The Nextera DNA Flex Library Prep kit reagents are not listed in the consumables document so they have been added to Table 1 below.

Your lab will most likely have everything already for their PulseNet activities. The exception to this is PhiX which will be listed in the appropriate step.

Nextera DNA Flex Library Prep Reference Guide

Nextera DNA Flex Library Prep Checklist

Nextera DNA Flex Library Prep Consumables and Equipment

Index Adapters Pooling Guide

Citation: Joel Sevinsky, Arian Nassiri, Heather Blankenship, Erin Young, Kevin Libuit, Kelly Oakeson, Lauren Turner, StaPH-B Consortium (04/29/2020). SARS-CoV-2 Sequencing on Illumina MiSeq Using ARTIC Protocol: Part 2 - Illumina DNA Flex Protocol. https://dx.doi.org/10.17504/protocols.io.bffyjjpw

Nextera DNA Flex Library Prep
by Illumina, Inc.
Catalog #: 20018705

Box #	Reagent	Description	Lot #	Exp. Date
1	SPB	Sample Purification Beads		
1	TSB	Tagment Stop Buffer		
1	TWB	Tagment Wash Buffer		
2	RSB	Resuspension Buffer		
2	TB1	Tagmentation Buffer 1		
2	EPM	Enhanced PCR Mix		
3	BLT	Bead-Linked Transposome		

Table 1: Reagent List from Nextera DNA Flex Library Prep Kit

DNA Flex - Dilution Plate Preparation

2 Dilution Plate Preparation Date/Initials:_____

Prior to starting your DNA Flex library prep, samples should be diluted into a 96 well plate as described below. Ideally, you would like each sample to be diluted such that the **30 µl** final volulme of sample contains **100 ng** to **500 ng** of DNA. Less than **100 ng** of DNA may cause the sample to be under represented in the pool, and modifications to increase the concentration of that sample are not practical when multiplexing the library prep. Much of this protocol can be achieved using 96 well plates, so having specimens organized in columns allows the use of an 8 channel multi-channel pipette.

2.1 [ ] Label a 96 well dilution plate with the number of columns required.

2.2 [ ] Add enough DNA to reach a total of at least **100 ng** ** and add molecular grade water (CED) to bring the total volume to **30 µl** .

NOTE: Preferred amount is **100 ng to **500 ng** . Less than that can lead to under representation of the sample in the final pool. More is probably ok as long as it is not extreme.

DNA Flex - Tagmentation 40m

3 Tagmentation Date/Initials:_____

40m

Citation: Joel Sevinsky, Arian Nassiri, Heather Blankenship, Erin Young, Kevin Libuit, Kelly Oakeson, Lauren Turner, StaPH-B Consortium (04/29/2020). SARS-CoV-2 Sequencing on Illumina MiSeq Using ARTIC Protocol: Part 2 - Illumina DNA Flex Protocol. https://dx.doi.org/10.17504/protocols.io.bffyjjpw

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

3.1 [ ] Prepare tagmenation Master Mix using **Calculation 1:**

Reagent	Volume (uL)	* (#samples +2)
BLT**	10.0	
TB1**	10.0	

Calculation 1: Tagmentation Master Mix

**NOTE: Vortex BLT prior to using. Both reagents should be at room temperature before using.

3.2 [ ] Vortex master mix, and then add **20 µl** to each sample well. Pipette each 10 times to mix.

3.3 [ ] Cover and seal the plate with Microseal 'B' (CED).

3.4 [ ] Place the plate in the thermal cycler and run the **TAG Program** below: 55°C

Step	Temp	Time
Tagmentation	55°C	15 min
Hold	10°C	Hold

TAG Program: Thermal cycler should be set for 50 uL volume and lid set to 105℃.

DNA Flex - Post Tagmentation Clean-up 45m

4 **Post Tagmentation Clean-up** Date/Initials:_____ 45m

This step stops the tagmentation and washes the adapter-tagged DNA on the BLT before PCR amplification. All reagents should be at **Room temperature**

4.1 [ ] Check **TSB** for precipitates, and then add **10 µl** to each tagmentation reaction (sample well). Slowly pipette to mix 10 times to resuspend the beads.

4.2 [ ] Seal the plate with Microseal 'B' (CED), place on the preprogrammed thermal cycler, and run the **PTC Program**.

Citation: Joel Sevinsky, Arian Nassiri, Heather Blankenship, Erin Young, Kevin Libuit, Kelly Oakeson, Lauren Turner, StaPH-B Consortium (04/29/2020). SARS-CoV-2 Sequencing on Illumina MiSeq Using ARTIC Protocol: Part 2 - Illumina DNA Flex Protocol. https://dx.doi.org/10.17504/protocols.io.bffyjjpw

Step	Temp	Time
Tagemntation Stop	37°C	15 min
Cool	10°C	Hold

PTC Program: Thermal cycler should be set for 60 uL volume and lid set to 105℃.

4.3 [ ] Place plate on magnet **00:03:00** or until clear.

4.4 [ ] Remove and discard supernatant.

4.5 [ ] Remove from magnet, and add **100 µl** **TWB**. Pipette to mix**.

NOTE: A deliberately slow pipetting technique minimizes the potential of **TWB foaming to avoid incorrect volume aspiration and incomplete mixing.

4.6 [ ] Place back on magnet **00:03:00** or until clear. Remove and discard supernatant.

4.7 [ ] Repeat **TWB** washes described above 2 more times. **Wait to discard supernatant and leave in magnet after second wash**.

DNA Flex- Amplify Tagmented DNA 45m

45m

5 Amplify Tagmented DNA Date/Initials:_____

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.

5.1 [ ] Prepare PCR master mix using **Calculation 2:**

Reagent	Volume (uL)	* (#samples +2)
EPM	20.0	
H2O	20.0	

Calculation 2: PCR Master Mix

5.2 [ ] Vortex and quick spin master mix.

Citation: Joel Sevinsky, Arian Nassiri, Heather Blankenship, Erin Young, Kevin Libuit, Kelly Oakeson, Lauren Turner, StaPH-B Consortium (04/29/2020). SARS-CoV-2 Sequencing on Illumina MiSeq Using ARTIC Protocol: Part 2 - Illumina DNA Flex Protocol. https://dx.doi.org/10.17504/protocols.io.bffyjjpw

5.3 [ ] Remove and discard **TWB** from samples. Remove from magnet, and immediately add **40 µl** PCR master mix, pipette to mix.

5.4 [ ] Add **10 µl** indices**, and then pipette to mix.

****NOTE: Use the same index strategy that you use for PusleNet organisms. If you are unfamiliar with the PulseNet protocol, see Index Adapters Pooling Guide for guidance on the indexing. A separate Appendix will be created discussing pooling best practices.**

5.5 [ ] Seal the plate with **Microseal 'B'** (CED), place on the thermal cycler, and run the **BLT PCR Program** on the thermal cycler.

Step	Temp	Time	Cycles
Elute	68°C	03:00	1
Denature	98°C	03:00	1
Denature	98°C	00:45	5**
Anneal	62°C	00:30	5**
Extend	68°C	02:00	5**
Extend	68°C	01:00	1
Hold	10°C	Hold	1

BLT PCR Program: Volume should be set for 50 uL and the lid temperature set to 105°C.

****NOTE: This is a 5 cycle PCR reaction designed for the majority of samples in 96 well plate sequencing. If your samples started with less than 100 ng of DNA, or eventually failed for low coverage in your pool, refer to page 10 of the Nextera DNA Flex Library Prep Reference Guide for guidelines on how to increase the number of cycles for low input samples.**

DNA Flex - Clean-up Libraries 50m

6 Clean-up Libraries Date/Initials:_____ 50m

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

6.1 [ ] Centrifuge plate at **280 x g, Room temperature 00:01:00** , and then place on magnet for **00:05:00** or until clear

6.2 [ ] Transfer **45 µl** of supernatant to clean wells of a new midi plate (CED), and then remove from magnet

Citation: Joel Sevinsky, Arian Nassiri, Heather Blankenship, Erin Young, Kevin Libuit, Kelly Oakeson, Lauren Turner, StaPH-B Consortium (04/29/2020). SARS-CoV-2 Sequencing on Illumina MiSeq Using ARTIC Protocol: Part 2 - Illumina DNA Flex Protocol. https://dx.doi.org/10.17504/protocols.io.bffyjjpw

6.3 [ ] Vortex and invert **SPB**.

6.4 [ ] Add **81 µl** of **SPB**** to samples, pipette 10

> ****NOTE: Since we are using this kit on small amplicons from the ARTIC Protocol, we will follow the DNA Flex instructions for small amplicon clean up, not standard DNA input.**

times to mix.

6.5 [ ] Seal (CED) plate and incubate for at least **00:05:00** .

6.6 [ ] Prepare fresh **80 % volume** EtOH using the following calculation:

0.4 ml * (#samples+1) = _____ mL volume EtOH

0.1 ml * (#samples+1) = _____mL volume molecular grade water

Add those two volumes together for **80 % volume** EtOH.

6.7 [ ] Place on magnet for **00:05:00** or until clear. Remove and discard supernatant.

6.8 [ ] Perform two **00:00:30** washes with **200 µl** **80 % volume** EtOH. Remove and discard supernatant after each wash. After second wash make sure to remove residual EtOH with 20 uL pipette. Perform this step on the magnetic stand without disturngin the beads.

6.9 [ ] Air dry beads approx. **00:05:00** and then remove plate from magnet

6.10 [ ] Vortex **RSB** and add **32 µl** . Pipette to mix and incubate at **Room temperature** for **00:02:00** .

6.11 [ ] Place plate on magnet for **00:02:00** . Transfer **30 µl** supernatant to new wells.

6.12 [ ] Library is now ready for quantification.

> **SAFE STOPPING POINT**

Citation: Joel Sevinsky, Arian Nassiri, Heather Blankenship, Erin Young, Kevin Libuit, Kelly Oakeson, Lauren Turner, StaPH-B Consortium (04/29/2020). SARS-CoV-2 Sequencing on Illumina MiSeq Using ARTIC Protocol: Part 2 - Illumina DNA Flex Protocol. https://dx.doi.org/10.17504/protocols.io.bffyjjpw

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.

DNA Flex - Qubit Quantification

7 Qubit Quantification Date/Initials:_____

7.1 [ ] Prepare Qubit working solution using **Calculation 5**. Label assay tubes for samples and standards.

Reagent	Volume (uL)	*(#rxns+2std+2)	Lot#	Exp. Date
Qubit Reagent	1.0			
Qubit Buffer	199.0			
Total	200.0			

Calculation 5: Qubit Working Solution

7.2 [ ] Combine **190 µl** Qubit working solution + **10 µl** Qubit standard into labeled standard tubes

7.3 [ ] Combine **198 µl** Qubit working solution + **2 µl** extracted DNA into labeled tubes

7.4 [ ] Vortex all tubes for 2-3 sec and incubate at **Room temperature** for minimum of **00:02:00** . Read tubes within **01:00:00**

7.5 [ ] Record sample results.

DNA Flex - Denaturation of Pooled Library

8 Denaturation of Pooled Library Date/Initials:_____

This section demonstrates how to generate a pooled library for V3 reagents on the MiSeq.

8.1 [ ] Centrifuge plate at **280 x g, Room temperature 00:01:00**

8.2 [ ] Make **4 Nanomolar (nM)** pool using **Calculation 6**

Citation: Joel Sevinsky, Arian Nassiri, Heather Blankenship, Erin Young, Kevin Libuit, Kelly Oakeson, Lauren Turner, StaPH-B Consortium (04/29/2020). SARS-CoV-2 Sequencing on Illumina MiSeq Using ARTIC Protocol: Part 2 - Illumina DNA Flex Protocol. https://dx.doi.org/10.17504/protocols.io.bffyjjpw

Pool conc. (Qubit value, ng/µl)	
Molarity (nM)= (Pool conc. x 1000) / 528	
Volume of pool to dilute (µl)= (4 x 50) / Molarity	
Volume of RSB to dilute (µl)= (50 – Pool to dilute)	

Calculation 6: 4nM Pool

8.3 [ ] Add **400 µl** molecular grade H_20 to the **100 µl** 1.0 N NaOH aliquot for 0.2 N NaOH

8.4 [ ] Combine **5 µl** 0.2 N NaOH and **5 µl** pooled DNA in a **1.5 ml** tube. Incubate **00:05:00** at **Room temperature**

8.5 [ ] Immediately add **990 µl** pre-chilled HT1. Pipette to mix, then use Table 2 for desired library pool concentration

Final Library Conc (pM)	8	10	12	14	15	16	18	20
Pooled Library (uL)	400.0	500.0	600.0	700.0	750.0	800.0	900.0	1000.0
HT1 (uL)	600.0	500.0	400.0	300.0	250.0	200.0	100.0	0.0

Table 2: Pooled Library Dilution - Calculation for 4 nM pooled library/20 pM denatured pooled library

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