Version 1

Jun 17, 2020

Illumina Nextera DNA Flex library construction and sequencing for SARS-CoV-2: Adapting COVID-19 ARTIC protocol V.1

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2 *Works for me* dx.doi.org/10.17504/protocols.io.bhjgj4jw

Coronavirus Method Development Community KRISP

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ABSTRACT

This protocol describes the procedure for generating cDNA from SARS-CoV-2 viral nucleic acid extracts and subsequently producing amplicons tiling the viral genome sequencing. It uses the V3 nCov-2019 primers from the ARTIC network. This is followed by library construction using Nextera Flex, which we found to save 9h of hands on time as compared with original protocol that use TrueSeq for library contstruction. It also describes the pooling of samples and quantitation, prior to sequencing on the Illumina Miseq and NextSeq.

It is adapted from the nCov-2019 sequencing protocol from Quick and colleagues, which can be found here:

Josh Quick. nCoV-2019 sequencing protocol.
http://dx.doi.org/10.17504/protocols.io.bdp7i5rn

EXTERNAL LINK

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THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Pillay S, Giandhari J, Tegally H, Wilkinson E, Chimukangara B, Lessells R, Mattison S, Moosa Y, Gazy I, Fish M, Singh L, Khanyile KS, Fonseca V, Giovanetti M, Alcantara LCJ, de Oliveira T. Whole Genome Sequencing of SARS-CoV-2: Adapting Illumina Protocols for Quick and Accurate Outbreak Investigation During a Pandemic. bioRxiv, doi: https://doi.org/10.1101/2020.06.10.144212 (2020).

DOI

dx.doi.org/10.17504/protocols.io.bhjgj4jw

PROTOCOL CITATION

Sureshnee Pillay, Jennifer Giandhari, Houriiyah Tegally, Eduan Wilkinson, Benjamin Chimukangara, Richard

Citation: Sureshnee Pillay, Jennifer Giandhari, Houriiyah Tegally, Eduan Wilkinson, Benjamin Chimukangara, Richard Lessells, Yunus Moosa, Inbal Gazy, Maryam Fish, Lavanya Singh, Khulekani Sedwell Khanyile, Vagner Fonseca, Marta Giovanetti, Luiz Carols Alcantara, Tulio de Oliveira, Jennifer Giandhari (06/17/2020). Illumina Nextera DNA Flex library construction and sequencing for SARS-CoV-2:ÃÂ Adapting COVID-19 ARTIC protocol. https://dx.doi.org/10.17504/protocols.io.bhjgj4jw

Lessells, Yunus Moosa, Inbal Gazy, Maryam Fish, Lavanya Singh, Khulekani Sedwell Khanyile, Vagner Fonseca, Marta Giovanetti, Luiz Carols Alcantara, Tulio de Oliveira, Jennifer Giandhari 2020. Illumina Nextera DNA Flex library construction and sequencing for SARS-CoV-2: Adapting COVID-19 ARTIC protocol. **protocols.io**
dx.doi.org/10.17504/protocols.io.bhjgj4jw

MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

Pillay S, Giandhari J, Tegally H, Wilkinson E, Chimukangara B, Lessells R, Mattison S, Moosa Y, Gazy I, Fish M, Singh L, Khanyile KS, Fonseca V, Giovanetti M, Alcantara LCJ, de Oliveira T. Whole Genome Sequencing of SARS-CoV-2: Adapting Illumina Protocols for Quick and Accurate Outbreak Investigation During a Pandemic. bioRxiv, doi: https://doi.org/10.1101/2020.06.10.144212 (2020).

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CREATED

Jun 16, 2020

LAST MODIFIED

Jun 17, 2020

OWNERSHIP HISTORY

Jun 16, 2020 Anita Broellochs protocols.io

Jun 17, 2020 Jennifer Giandhari

PROTOCOL INTEGER ID

38216

PARENT PROTOCOLS

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GUIDELINES

Introduction

This protocol describes a method for whole genome sequencing of the SARS-CoV-2 using a tiling PCR approach with overlapping primers and Nextera Flex libraries for Illumina sequencers. This method was produced in KRISP labs for the Network of Genomics Surveillance of South Africa (NGS-SA).

Briefly, primers are designed to be 20-30bp in length and to generate 400bp amplicons with a 70bp overlap. The primers are designed using an online tool called Primal Scheme (http://primal.zibraproject.org/).The amplicons generated can be sequenced on the on the Illumina MiSeq. This will produce next generation sequences covering the whole genome of the SARS-CoV-2 .

Purpose

The purpose of this document is to provide detailed instructions that should be followed when performing the sequencing of SARS-CoV-2 whole genomes from RNA samples using the Nextera DNA Flex Library Kit.

MATERIALS

NAME	CATALOG #	VENDOR
Q5 Hot Start High-Fidelity DNA Polymerase - 100 units	M0493S	New England Biolabs
Qubit™ Assay Tubes	Q32856	Invitrogen - Thermo Fisher
Qubit dsDNA HS Assay kit	Q32854	Thermo Fisher Scientific
SuperScript™ IV Reverse Transcriptase	18090050	Thermo Fisher Scientific
Random Hexamers (50 µM)	N8080127	Thermo Fisher
dNTP Mix (10 mM each)	R0192	Thermo Fisher
AMPure XP	A63881	Beckman Coulter
RNaseOUT Recombinant Ribonuclease Inhibitor	10777019	Thermo Fisher Scientific
Artic Primers-specific for 2019-nCoV according to Primal Scheme		
Nextera DNA Flex Library Prep Kit		Illumina, Inc.
Nextera™ DNA UD Indexes (96 Indexes 96 Samples)		
MiSeq Reagent Nano Kit v2 (500 cycles)	MS-103-1003	Illumina, Inc.
DNA High Sensitivity Reagent Kit	CLS760672	Perkin Elmer
DNA 1K / 12K / Hi Sensitivity Assay LabChip	760517	Perkin Elmer
General PCR laboratory equipment and consumables		

cDNA

1 Prepare the cDNA mastermix in the pre-PCR clean room. The mastermix hood must be decontaminated before and after use with 10% extran, and 70% ethanol, and sterilised with ultraviolet light (UV).

2 

Mix the following components in a labeled 1.5ml Component:

Component	Volume (ul)
50µM Random Hexamers 1	1
10mM dNTPs mix (10mM each) 1	1
Template RNA	11
Total	*12*

Table 1. cDNA synthesis mastermix 1

2.1 Add **1 µl 50µM Random Hexamers 1** to a labeled 1.5ml eppendorf tube.

2.2 Add **1 µl 10mM dNTPs mix (10mM each) 1** .

Citation: Sureshnee Pillay, Jennifer Giandhari, Houriiyah Tegally, Eduan Wilkinson, Benjamin Chimukangara, Richard Lessells, Yunus Moosa, Inbal Gazy, Maryam Fish, Lavanya Singh, Khulekani Sedwell Khanyile, Vagner Fonseca, Marta Giovanetti, Luiz Carols Alcantara, Tulio de Oliveira, Jennifer Giandhari (06/17/2020). Illumina Nextera DNA Flex library construction and sequencing for SARS-CoV-2:ÃÂ Adapting COVID-19 ARTIC protocol. https://dx.doi.org/10.17504/protocols.io.bhjgj4jw

2.3 Add **11 µl Template RNA** .

The total volume in the tube should now be **13 µl** .

3 

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

4 Aliquot the mastermix in labelled PCR strip tubes.

PCR master mixes (shown in Tables 1 and 3) can be prepared at the same time, in the pre-PCR area before starting amplifications.

5 

Incubate the reaction as follows in a thermal cycler.

Temperature (°C)	Time
65	5 minutes
4	1 minute

Table 2. PCR conditions

6 Spin down the tubes with the RNA and primers to get all liquid to the bottom.

7 

Prepare the following mastermix in the clean mastermix room.

Mix the following components in a labeled 1.5ml eppendorf tube:

Component	Volume (µl)
SSIV Buffer	4
100mM DTT	1
RNaseOUT RNase Inhibitor	1
SSIV Reverse Transcriptase	1
Total	*7*

Table 3. cDNA synthesis mastermix 2

7.1 Add **4 µl SSIV Buffer** to a labeled 1.5ml eppendorf tube.

Citation: Sureshnee Pillay, Jennifer Giandhari, Houriiyah Tegally, Eduan Wilkinson, Benjamin Chimukangara, Richard Lessells, Yunus Moosa, Inbal Gazy, Maryam Fish, Lavanya Singh, Khulekani Sedwell Khanyile, Vagner Fonseca, Marta Giovanetti, Luiz Carols Alcantara, Tulio de Oliveira, Jennifer Giandhari (06/17/2020). Illumina Nextera DNA Flex library construction and sequencing for SARS-CoV-2:ÃÂ Adapting COVID-19 ARTIC protocol. https://dx.doi.org/10.17504/protocols.io.bhjgj4jw

7.2 Add **1 µl 100mM DTT** .

7.3 Add **1 µl RNaseOUT RNase Inhibitor** .

7.4 Add **1 µl SSIV Reverse Transcriptase** .

The total volume should now be **7 µl** .

8 

The mastermix must be added to the **13 µl denatured RNA** for a **20 µl total volume** .

9 

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

10 

Incubate the reaction as follows in a thermal cycler.

Temperature (°C)	Time
42	50 minutes
70	10 minutes
5	Hold

Table 4. PCR conditions

Primer Pool Preparation

11 Primers must be diluted and pooled using nuclease free water in a clean mastermix hood. The mastermix hood must be decontaminated before and after use with 10% extran, and 70% ethanol, and sterilised with ultraviolet light (UV).

12 

If required, resuspend lyophilised primers at a concentration of **[M]100 µM** each.

2019- nCoV primers for this protocol were designed using Primal Scheme to generate overlapping 400 nucleotide amplicons.

Citation: Sureshnee Pillay, Jennifer Giandhari, Houriiyah Tegally, Eduan Wilkinson, Benjamin Chimukangara, Richard Lessells, Yunus Moosa, Inbal Gazy, Maryam Fish, Lavanya Singh, Khulekani Sedwell Khanyile, Vagner Fonseca, Marta Giovanetti, Luiz Carols Alcantara, Tulio de Oliveira, Jennifer Giandhari (06/17/2020). Illumina Nextera DNA Flex library construction and sequencing for SARS-CoV-2:ÃÂ Adapting COVID-19 ARTIC protocol. https://dx.doi.org/10.17504/protocols.io.bhjgj4jw

13

To generate **100 µM primer pool stocks** , add **5 µl of each primer pair** (named pool 1 or pool 2) to a 1.5ml eppendorf tube labeled either "***Pool 1 (100µM)***" or "***Pool 2 (100µM)***".

Total volume will be **490 µl** for Pool 1 (100uM) and **490 µl** for Pool 2 (100uM). These are now **100 µM** stocks of each primer.

14 Dilute the 100µM primer pool 1:10 in molecular grade water, to generate **10 µM primer stocks** .

It is recommended that multiple aliquots of each primer pool are made in case of degradation or contamination.

15

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 **3.6 µl primer pools (10µM)** per **25 µl reaction** .

Tiling PCR

16 Prepare the PCR mastermix in the clean mastermix room.

17 The mastermix hood must be decontaminated before and after use with 10% extran, and 70% ethanol, and sterilised with ultraviolet light (UV).

18

A mastermix for each pool must be made up in the mastermix hood.

Mix the following components in a labeled 1.5ml eppendorf tube:

Component	Pool 1 volumes (µl)	Pool 2 volumes (µl)
5X Q5 Reaction Buffer	5	5
10mM dNTPs	0.5	0.5
Q5 Hot Start DNA Polymerase	0.25	0.25

Citation: Sureshnee Pillay, Jennifer Giandhari, Houriiyah Tegally, Eduan Wilkinson, Benjamin Chimukangara, Richard Lessells, Yunus Moosa, Inbal Gazy, Maryam Fish, Lavanya Singh, Khulekani Sedwell Khanyile, Vagner Fonseca, Marta Giovanetti, Luiz Carols Alcantara, Tulio de Oliveira, Jennifer Giandhari (06/17/2020). Illumina Nextera DNA Flex library construction and sequencing for SARS-CoV-2:ÃÂ Adapting COVID-19 ARTIC protocol. https://dx.doi.org/10.17504/protocols.io.bhjgj4jw

Primer Pool 1 or 2 (10µM)	3.6	3.6
Nuclease-free water	10.65	10.65
Total	*20*	*20*

Table 5. PCR mastermix

18.1 Add **5 µl Table 5. PCR mastermix** to a labeled 1.5ml eppendorf tube.

18.2 Add **0.5 µl 10mM dNTPs** .

18.3 Add **0.25 µl Q5 Hot Start DNA Polymerase** .

18.4 Add **3.6 µl Primer Pool 1 or 2 (10µM)** .

18.5 Add **10.65 µl Nuclease-free water** .

The total volume should now be **20 µl** .

19 Aliquot the mastermix in labelled PCR strip tubes.

20 Add **5 µl of cDNA** under the extraction hood or general lab hood, which has been decontaminated using with 10% extran, and 70% ethanol, and sterilised with ultraviolet light (UV).

21 

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

22 Incubate the reaction as follows in a thermal cycler.

Step	Temperature (°C)	Time	Cycles
Heat Activation	98	30 seconds	1
Denaturation	98	15 seconds	35
Annealing	65	5 minutes	
Hold	4	∞	

Table 6. PCR conditions

*Cycle number should be 25 for Ct 18-21, and up to a maximum of 35 cycles for Ct 35.

Citation: Sureshnee Pillay, Jennifer Giandhari, Houriiyah Tegally, Eduan Wilkinson, Benjamin Chimukangara, Richard Lessells, Yunus Moosa, Inbal Gazy, Maryam Fish, Lavanya Singh, Khulekani Sedwell Khanyile, Vagner Fonseca, Marta Giovanetti, Luiz Carols Alcantara, Tulio de Oliveira, Jennifer Giandhari (06/17/2020). Illumina Nextera DNA Flex library construction and sequencing for SARS-CoV-2:ÃÂ Adapting COVID-19 ARTIC protocol. https://dx.doi.org/10.17504/protocols.io.bhjgj4jw

PCR Clean-up

23 Combine the entire contents of "***Pool 1***" and "***Pool 2***" PCR reactions for each biological sample into a single 1.5 ml eppendorf tube.

24 Vortex Ampure beads thoroughly to ensure they are well resuspended; the solution should be a homogenous brown colour.

25 

Add an equal volume (1:1) of Ampure beads to the pooled sample tube and mix gently by either flicking or pipetting.

For example, add **50 µl Ampure beads** to a **50 µl reaction** .

26 Pulse centrifuge to collect all liquid at the bottom of the tube.

27 

Incubate for **00:05:00** at **Room temperature** .

28 Place on magnetic rack and incubate for **00:02:00** or until the beads have pelleted and the supernatant is completely clear.

29 Carefully remove and discard the supernatant, being careful not to touch the bead pellet.

30 

Add **200 µl of freshly prepared 70% ethanol** (at **Room temperature**) to the pellet.

31 Carefully remove and discard ethanol, being careful not to touch the bead pellet.

32 

Add **200 µl of freshly prepared 70% ethanol** (at **Room temperature**) to the pellet.

Citation: Sureshnee Pillay, Jennifer Giandhari, Houriiyah Tegally, Eduan Wilkinson, Benjamin Chimukangara, Richard Lessells, Yunus Moosa, Inbal Gazy, Maryam Fish, Lavanya Singh, Khulekani Sedwell Khanyile, Vagner Fonseca, Marta Giovanetti, Luiz Carols Alcantara, Tulio de Oliveira, Jennifer Giandhari (06/17/2020). Illumina Nextera DNA Flex library construction and sequencing for SARS-CoV-2:ÃÂ Adapting COVID-19 ARTIC protocol. https://dx.doi.org/10.17504/protocols.io.bhjgj4jw

33 Carefully remove and discard ethanol, being careful not to touch the bead pellet.

34

Pulse centrifuge to collect all liquid at the bottom of the tube and carefully remove as much residual ethanol as possible using a P10 pipette.

35

With the tube lid open incubate for **00:01:00** or until the pellet loses its shine.

If the pellet dries completely it will crack and become difficult to resuspend

36

Resuspend pellet in **30 µl Elution Buffer (EB)** , mix gently by either flicking or pipetting and incubate for **00:02:00** .

37 Place on magnetic stand and transfer sample to a clean 1.5mL eppendorf tube ensuring no beads are transferred into this tube.

38

***Sample concentration can be determined using the Qubit and the size of amplicons can be visualized using the LabChip Fragment Analyzer.

The expected amplicon size is 400bp.

Library Preparation Tagment Amplicon DNA

39

Item	Storage	Instructions
BLT(bead-linked transposomes)	2°C to 8°C	Bring to room temperature. Vortex to mix. Do not centrifuge before pipetting.

Citation: Sureshnee Pillay, Jennifer Giandhari, Houriiyah Tegally, Eduan Wilkinson, Benjamin Chimukangara, Richard Lessells, Yunus Moosa, Inbal Gazy, Maryam Fish, Lavanya Singh, Khulekani Sedwell Khanyile, Vagner Fonseca, Marta Giovanetti, Luiz Carols Alcantara, Tulio de Oliveira, Jennifer Giandhari (06/17/2020). Illumina Nextera DNA Flex library construction and sequencing for SARS-CoV-2:ÃÂ Adapting COVID-19 ARTIC protocol. https://dx.doi.org/10.17504/protocols.io.bhjgj4jw

TB1(Tagmentation buffer)	-25°C to -15°C	Bring to room temperature. Vortex to mix.

Table 7. Preparation of reagents

40 

Add **2 µl** – **30 µl DNA** to each well of a 96-well PCR plate / 0.2ml strip tubes so that the total input amount is 100–500 ng.

41 

If DNA volume < 30 µl, add nuclease-free water to the DNA samples to bring the total volume to 30 µl.

42 Vortex BLT vigorously for **00:00:10** to resuspend.

43 Vortex in between adding BLT as necessary.

44 Prepare the tagmentation master mix.

Multiply each volume by the number of samples being processed

Component	Volume (µl) per sample
BLT	11
TB1	11
Total	22

Table 8. Tagmentation Master Mix

44.1 Please scale this step as needed.

For **1 sample** :

11 µl BLT

11 µl TB1

---

22 µl Total

45 

Vortex the tagmentation master mix thoroughly.

Citation: Sureshnee Pillay, Jennifer Giandhari, Houriiyah Tegally, Eduan Wilkinson, Benjamin Chimukangara, Richard Lessells, Yunus Moosa, Inbal Gazy, Maryam Fish, Lavanya Singh, Khulekani Sedwell Khanyile, Vagner Fonseca, Marta Giovanetti, Luiz Carols Alcantara, Tulio de Oliveira, Jennifer Giandhari (06/17/2020). Illumina Nextera DNA Flex library construction and sequencing for SARS-CoV-2:ÃÂ Adapting COVID-19 ARTIC protocol. https://dx.doi.org/10.17504/protocols.io.bhjgj4jw

46

Transfer **20 µl tagmentation master mix** to each well of the plate containing a sample.

Use fresh tips for each sample column.

47

Resuspend by pipetting each sample 10 times.

48 Seal the plate with a plate sealer, place on the preprogrammed thermal cycler, and run the tagmentation program.

Temperature (°C)	Time
55	15 minutes
10	Hold

PCR – Tagmentation conditions

Post Tagmentation Clean-up

49

Item	Storage	Instructions
TSB (Tagment stop buffer)	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 (Tagment wash buffer)	15°C to 30°C	Use at room temperature.

Table 10. Preparation of Reagents

Temperature (°C)	Time
37	15 minutes
10	Hold

Table 11. Post Tagmentation Cleanup

50

Add **10 µl TSB** to the tagmentation reaction.

Citation: Sureshnee Pillay, Jennifer Giandhari, Houriiyah Tegally, Eduan Wilkinson, Benjamin Chimukangara, Richard Lessells, Yunus Moosa, Inbal Gazy, Maryam Fish, Lavanya Singh, Khulekani Sedwell Khanyile, Vagner Fonseca, Marta Giovanetti, Luiz Carols Alcantara, Tulio de Oliveira, Jennifer Giandhari (06/17/2020). Illumina Nextera DNA Flex library construction and sequencing for SARS-CoV-2:ÃÂ Adapting COVID-19 ARTIC protocol. https://dx.doi.org/10.17504/protocols.io.bhjgj4jw

51 

Resuspend the beads by slowly pipetting each well/ tube 10 times.

52 Seal the plate with / tubes, place on the preprogrammed thermal cycler, and run the post tagmentation cleanup program.

53 Place the plate on the magnetic stand for approximately **00:03:00** until liquid is clear.

54 Using a multichannel pipette, remove and discard supernatant.

55 

Remove the sample plate from the magnetic stand and use a deliberately slow pipetting technique to add **100 µl TWB** directly onto the beads.

This slow pipetting technique minimizes the potential of TWB foaming to avoid incorrect volume aspiration and incomplete mixing.

56 

Slowly pipette until beads are fully resuspended.

57 Place the plate on the magnetic stand for approximately **00:03:00** until liquid is clear.

58 

Using a multichannel pipette, remove and discard supernatant.

59 

Remove the plate from the magnetic stand and use a deliberately slow pipetting technique to add **100 µl TWB** directly onto the beads.

60 

Slowly pipette each well/tube to resuspend the beads.

61 Place the plate on the magnetic stand for approximately **00:03:00** until liquid is clear.

Citation: Sureshnee Pillay, Jennifer Giandhari, Houriiyah Tegally, Eduan Wilkinson, Benjamin Chimukangara, Richard Lessells, Yunus Moosa, Inbal Gazy, Maryam Fish, Lavanya Singh, Khulekani Sedwell Khanyile, Vagner Fonseca, Marta Giovanetti, Luiz Carols Alcantara, Tulio de Oliveira, Jennifer Giandhari (06/17/2020). Illumina Nextera DNA Flex library construction and sequencing for SARS-CoV-2:ÃÂ Adapting COVID-19 ARTIC protocol. https://dx.doi.org/10.17504/protocols.io.bhjgj4jw

62 

Using a multichannel pipette, remove and discard supernatant.

63 

Remove the plate from the magnetic stand and use a deliberately slow pipetting technique to add **100 µl TWB** directly onto the beads.

64 Slowly pipette each well/tube to resuspend the beads.

65 Seal the plate and keep on the magnetic stand until step 69 of the Procedure section in Amplify Tagmented DNA.

66 The TWB remains in the wells to prevent overdrying of the beads.

Amplify Tagment DNA

67

Item	Storage	Instructions
EPM (enhanced PCR MIx)	-25°C to -15°C	Thaw on ice. Invert to mix, then briefly centrifuge.
Index Adapters (Plates)	-25°C to -15°C	Thaw at room temperature. Spin briefly before use.

Table 12. Preparation of Reagents

Combine the following volumes to prepare the PCR master mix. Multiply each volume by the number of samples being processed.

Component	Volume (µl) per sample
EPM	22
NFW	22
Total	44

Table 13. PCR Master Mix

Citation: Sureshnee Pillay, Jennifer Giandhari, Houriiyah Tegally, Eduan Wilkinson, Benjamin Chimukangara, Richard Lessells, Yunus Moosa, Inbal Gazy, Maryam Fish, Lavanya Singh, Khulekani Sedwell Khanyile, Vagner Fonseca, Marta Giovanetti, Luiz Carols Alcantara, Tulio de Oliveira, Jennifer Giandhari (06/17/2020). Illumina Nextera DNA Flex library construction and sequencing for SARS-CoV-2:ÃÂ Adapting COVID-19 ARTIC protocol. https://dx.doi.org/10.17504/protocols.io.bhjgj4jw

Reagent overage is included in the volume to ensure accurate pipetting.

67.1 Please scale this step as needed.

For **1 sample** :

22 µl EPM

22 µl NFW

---

44 µl Total

68

Vortex and centrifuge the PCR master mix at **280 x g 00:00:10** .

69

With the plate on the magnetic stand, use a 200 µl multichannel pipette to remove and discard supernatant. (from step 65 of post tagmentation clean-up)

Foam that remains on the well walls does not adversely affect the library.

70 Remove from the magnet.

71

Immediately add **40 µl PCR master mix** directly onto the beads in each sample well/ tube.

72

Pipette mix until the beads are fully resuspended.

Alternatively, seal the plate and use a plate shaker at 1600 rpm for **00:01:00** .

Citation: Sureshnee Pillay, Jennifer Giandhari, Houriiyah Tegally, Eduan Wilkinson, Benjamin Chimukangara, Richard Lessells, Yunus Moosa, Inbal Gazy, Maryam Fish, Lavanya Singh, Khulekani Sedwell Khanyile, Vagner Fonseca, Marta Giovanetti, Luiz Carols Alcantara, Tulio de Oliveira, Jennifer Giandhari (06/17/2020). Illumina Nextera DNA Flex library construction and sequencing for SARS-CoV-2:ÃÂ Adapting COVID-19 ARTIC protocol. https://dx.doi.org/10.17504/protocols.io.bhjgj4jw

73

Seal the sample plate and centrifuge at **280 x g 00:00:03** .

74

Add **10 µl** of the appropriate index adapters to each sample.

75

Using a pipette set to 40 µl, pipette 10 times to mix.

Alternatively, seal the plate/ tubes and use a plate shaker at 1600 rpm for **00:01:00** .

76

Centrifuge at **280 x g 00:00:30** .

77 Place on the thermal cycler and run the Enrichment PCR program.

Temperature (°C)	Time	
68	3 minutes	
98	3 minutes	
98	45 seconds	8 cycles
62	30 seconds	
68	2 minutes	
68	1 minute	
10	Hold	

Table 13. PCR Conditions

78

SAFE STOPPING POINT

If you are stopping, store at **2 °C** to **8 °C** for up to **72:00:00** (3 days).

Clean-up Libraries

79

Item	Storage	Instructions

Citation: Sureshnee Pillay, Jennifer Giandhari, Houriiyah Tegally, Eduan Wilkinson, Benjamin Chimukangara, Richard Lessells, Yunus Moosa, Inbal Gazy, Maryam Fish, Lavanya Singh, Khulekani Sedwell Khanyile, Vagner Fonseca, Marta Giovanetti, Luiz Carols Alcantara, Tulio de Oliveira, Jennifer Giandhari (06/17/2020). Illumina Nextera DNA Flex library construction and sequencing for SARS-CoV-2:ÃÂ Adapting COVID-19 ARTIC protocol. https://dx.doi.org/10.17504/protocols.io.bhjgj4jw

SPB (Sample Purification Beads)	2°C to 8°C	Let stand at room temperature for 30 minutes. Vortex and invert to mix.
RSB (Resuspension Buffer)	-25°C to -15°C	Thaw and bring to room temperature . Vortex to mix.

Table 14. Preparation of Reagents

80 Prepare fresh [M]**80 % EtOH** from absolute ethanol.

81 

Centrifuge at **280 x g 00:01:00** to bring all contents to the bottom.

82 Place the plate/ tubes on a magnetic stand for approximately **00:05:00** until liquid is clear.

83 

Transfer **45 µl** supernatant from each well of the PCR plate/ tubes to the corresponding well of a new plate/ tubes.

84 Vortex and invert SPB multiple times to resuspend.

85 

Add **40 µl nuclease-free water** to each well/ tube.

86 

Add **45 µl SPB** to each well/ tube.

87 

Citation: Sureshnee Pillay, Jennifer Giandhari, Houriiyah Tegally, Eduan Wilkinson, Benjamin Chimukangara, Richard Lessells, Yunus Moosa, Inbal Gazy, Maryam Fish, Lavanya Singh, Khulekani Sedwell Khanyile, Vagner Fonseca, Marta Giovanetti, Luiz Carols Alcantara, Tulio de Oliveira, Jennifer Giandhari (06/17/2020). Illumina Nextera DNA Flex library construction and sequencing for SARS-CoV-2:ÃÂ Adapting COVID-19 ARTIC protocol. https://dx.doi.org/10.17504/protocols.io.bhjgj4jw

Mix well by pipetting 10 times.

Alternatively, seal the plate and use a plate shaker at 1600 rpm for **00:01:00** .

88

Incubate at **Room temperature** for **00:05:00** .

89 Place on the magnetic stand for approximately **00:05:00** until the liquid is clear.

During incubation, thoroughly vortex the SPB (undiluted stock tube), and then add **15 µl** to each well of a new plate/tubes.

90

Transfer **125 µl** supernatant from each well of the first plate/ tubes into the corresponding well of the second plate/ tubes (containing 15 µl undiluted SPB).

91

Mix well by pipetting 10 times.

Alternatively, seal the plate and use a plate shaker at 1600 rpm for **00:01:00** .

92 Discard the first plate/ tubes.

93

Pipette each well 10 times to mix.

94

Incubate at **Room temperature** for **00:05:00** .

95 Place on the magnetic stand for approximately **00:05:00** until the liquid is clear.

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96 Without disturbing the beads, remove and discard supernatant.

97 

Wash two times as follows:

97.1 

Add **200 µl freshly prepared 80% ethanol** with the plate on the magnetic stand.

97.2 

Incubate for **00:00:30** .

97.3 Without disturbing the beads, remove and discard the supernatant.

97.4 

Add **200 µl freshly prepared 80% ethanol** with the plate on the magnetic stand.

97.5 Without disturbing the beads, remove and discard the supernatant.

97.6 

Use a 20 µl pipette to remove any residual ethanol.

97.7 Air-dry on the magnetic stand for **00:05:00** .

97.8 Remove from the magnetic stand.

97.9 

Add **32 µl RSB** to each well/ tube.

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97.10 

Resuspend by pipette mixing.

97.11 

Incubate at **Room temperature** for **00:02:00** .

97.12 Place the plate/ tubes on the magnetic stand for approximately **00:02:00** .

97.13 Transfer **30 µl supernatant** to a new 96-well PCR plate/ tubes.

98 

SAFE STOPPING POINT

If you are stopping, seal the plate, and store at **-25 °C** to **-15 °C** for up to 30 days.

Normalization of DNA

99 

Quantify the DNA as described using the Qubit and determine the fragment length using the LabChip.

100 Using the Qubit concentrations and fragment length normalize the libraries to equimolar 4nM by diluting with RSB buffer.

101 

Calculate appropriate amount of diluent in an excel sheet to add to respective sample libraries in order to achieve a 4nm library concentration, using the following formula: Nanomolar concentration = (ng/µl /660 x 500) x 10^6

102 

Pipette mix 5 times.

103 

Use a multi-channel pipette to transfer **5 µl of the diluted sample library** to an 8 strip-tube and spin briefly.

104 Pool the library samples from the 8-strip tubes to a labelled Pooled Amplicon Library (PAL) 2ml eppendorf tube.

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105 Proceed to library denaturation.

Library Denaturation

106 Remove the tube of HT1 (Hybridization Buffer) from the freezer (-15°C to -25°C) and set aside at **Room temperature** to thaw.

107 When thawed, store at **2 °C** to **8 °C** until you are ready to dilute denatured libraries.

108 

Prepare **500µl of 0.2 N NaOH** by combining the following volumes in a 1.5ml microcentrifuge tube:
490 µl laboratory-grade water and **10 µl Stock 1.0 N NaOH** .

Refer to the formula below:

$$1M = 1N$$
$$10N(x) = (0.2)(500)$$

x = **10 µl NaOH** + **490 µl laboratory-grade water**

A fresh dilution of 0.2N NaOH is required for the denaturation process in preparing sample DNA and a PhiX control.

109 

Invert the tube several times to mix.

110 

Combine the following volumes of pooled sample DNA and freshly diluted 0.2 N NaOH in a micro-centrifuge tube, by adding **5 µl of 4nM sample DNA** to **5 µl of 4nM sample DNA** .

111 Discard the remaining dilution of 0.2 N NaOH or set aside to prepare a PhiX control within the next **12:00:00** .

112 

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Vortex briefly to mix the sample solution, and then centrifuge the sample solution to **280 x g 00:01:00** .

113

Incubate for **00:05:00** at **Room temperature** to denature the DNA into single strands.

114

Add **10 µl of 4nM sample DNA** to **990 µl of pre-chilled HT1** .

The result is a 20pM denatured library in 1 mM NaOH.

115 Place the denatured DNA **On ice** or at **4 °C** until you are ready to proceed to the final dilution.

Dilution of Denatured Library

116 Use the following instructions to dilute the 20pM DNA further to give 600µl of the desired input concentration.

Dilute the denatured DNA to the desired concentration using the following example (if using 5% PhiX):

Final Concentration	20pM denatured DNA	5% PhiX	Pre-chilled HT1
12pM	356.4ul	3.6ul	240ul

*This was found to be the optimal loading concentration when using a Miseq V2 Nano 500 cycle kit

117

Invert several times to mix and then pulse centrifuge.

118

To dilute PhiX to 4nM concentration, combine the following volumes in a microcentrifuge tube:

- **2 µl of 10nM PhiX library**
- **3 µl of 10mM Tris-Cl** , pH **8.5** with [M] **0.1 % Tween 20**

If not prepared within the last 12 hours, prepare a fresh dilution of 0.2 N NaOH.

119

Combine the following volumes in a micro-centrifuge tube:

- **5 µl of 4 nM PhiX library**

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- **5 µl of 0.2 N NaOH**

120 

Vortex briefly to mix.

121 

Centrifuge at **280 x g 00:01:00** .

122 

Incubate at **Room temperature** for **00:05:00** .

123 

Dilute denatured PhiX to 20pM by adding pre-chilled HT1 to the denatured PhiX library as follows:

- **10 µl denatured PhiX library**
- **990 µl pre-chilled HT1**

124 

Invert to mix.

125 Combine Library and PhiX Control.

126 

Mix this solution well and briefly centrifuge.

127 Keep **On ice** or at **4 °C** until it is ready to be loaded onto the MiSeq reagent cartridge.

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