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

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Coronavirus Method De... 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:

Citation

Josh Quick (2026). nCoV-2019 sequencing protocol.

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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

MATERIALS

- Q5 Hot Start High-Fidelity DNA Polymerase - 100 units **New England Biolabs** **Catalog #**M0493S
- Qubit™ Assay Tubes **Invitrogen - Thermo Fisher** **Catalog #**Q32856
- Qubit dsDNA HS Assay kit **Thermo Fisher Scientific** **Catalog #**Q32854
- SuperScript™ IV Reverse Transcriptase **Thermo Fisher Scientific** **Catalog #**18090050
- Random Hexamers (50 µM) **Thermo Fisher** **Catalog #**N8080127
- dNTP Mix (10 mM each) **Thermo Fisher** **Catalog #**R0192
- AMPure XP **Beckman Coulter** **Catalog #**A63881
- RNaseOUT Recombinant Ribonuclease Inhibitor **Thermo Fisher Scientific** **Catalog #**10777019
- 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) **Illumina, Inc.** **Catalog #**MS-103-1003
- DNA High Sensitivity Reagent Kit **Perkin Elmer** **Catalog #**CLS760672
- DNA 1K / 12K / Hi Sensitivity Assay LabChip **Perkin Elmer** **Catalog #**760517
- 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 .

2.3 Add 11 µL Template RNA .

Note

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.

Note

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.

7.2 Add 1 µL 100mM DTT .

7.3 Add 1 µL RNaseOUT RNase Inhibitor .

7.4 Add 1 µL SSIV Reverse Transcriptase .

> Note
>
> 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.

Note

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

13 To generate [M] 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)***".

Note

Total volume will be 490 µL for Pool 1 (100uM) and 490 µL for Pool 2 (100uM). These are now [M] 100 µM stocks of each primer.

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

Note

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

15

Note

Primers need to be used at a final concentration of [M] 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
	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 .

Note

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.

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.

Note

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.

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.

Note

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.

Expected result

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

			ex 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.

46 Transfer 20 µL tagmentation master mix to each well of the plate containing a sample.

Note

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.

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.

Note

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.

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

Note

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)

Note

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.

Note

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

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.

Note

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
	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 Mix well by pipetting 10 times.

> Note
>
> 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.

Note

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.

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.

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 × 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.

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

Note

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 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 .

Note

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

Note

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
- 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.

Citations

Josh Quick. nCoV-2019 sequencing protocol
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