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NEBnext library construction and sequencing for SARS-CoV-2: Adapting COVID-19 ARTIC protocol

Forked from Illumina Nextera DNA Flex library construction and sequencing for SARS-CoV-2: Adapting COVID-19 ARTIC protocol

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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 uses TruSeq for library contstruction. It also describes the pooling of samples and quantitation, prior to sequencing on the Illumina Miseq.

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

CITATION

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

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 NEBNext Ultra II Library Preparation Kit 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 NEBNext Ultra II Library Preparation 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
- 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
- NEBNext Ultra II Library Preparation Kit **New England Biolabs** **Catalog #**NEB #E7103
- NEBNext Multiplex Oligos **New England Biolabs** **Catalog #**E6442S

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	*13*

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 pool.

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 5X Q5 Reaction Buffer 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 End Repair

39

Note

For a 400bp insert, use 200ng input DNA. Input amounts lower than those specified results in low yield and increased duplicates

Note

Starting Material: *Cleaned-up DNA diluted to 1 – 5 ng/µl, in at least 50 µl volume.*

40 Prepare the end repair master mix.

Multiply each volume by the number of samples being processed

	Component	Volume (µl) per sample
	NEBNext Ultra II End Prep Enzyme Mix (green top)	3
	NEBNext Ultra II End Prep Reaction Buffer (green top)	7
	Total	10

Table 8. End Repair Master mix

40.1 Please scale this step as needed.

For 50 sample :

3 µL NEBNext Ultra II End Prep Enzyme Mix (green top)

7 µL NEBNext Ultra II End Prep Reaction Buffer (green top)

---

60 µL Total

41 Add 50µl of DNA to the respective wells, for a total reaction volume of 60 µl.

42 Mix well on a vortex mixer or by pipetting up and down 10 times.

43 Perform a quick spin to collect all liquid from the sides of the tube.

Note

It is important to mix well. The presence of a small amount of bubbles will not interfere with performance.

44 Place in a thermocycler, with the heated lid set to ≥75°C, and run the following program:

45 Seal the plate with a plate sealer, place on the preprogrammed thermal cycler, and run the end repair program.

	Temperature (°C)	Time
	20	30 minutes
	65	30 minutes
	10	Hold

PCR – End repair conditions

Note

If necessary, samples can be stored at –20°C; however, a slight loss in yield (~20%) may be observed. We recommend continuing with adaptor ligation before stopping.

Adapter Ligation

46 Make up the following Mastermix and add 31µl to the respective wells.

47 Add 35µl of the End Prep DNA to the respective wells.

48 Add 2.5µl of NEBNext Adpaters for Illumina to the respective wells.

49 Mix well on a vortex mixer or by pipetting up and down 10 times.

50 Perform a quick spin to collect all liquid from the sides of the tube. Incubate at 20°C for 15 minutes in a thermocycler with the heated lid **open**.

Note

The Ligation Master Mix and Ligation Enhancer can be mixed ahead of time and is stable for at least 8 hours @ 4°C. We do not recommend adding adaptor to a premix in the Adaptor Ligation Step.

Note

The NEBNext Ultra II Ligation Master Mix is very viscous. Care should be taken to ensure adequate mixing of the ligation reaction, as incomplete mixing will result in reduced ligation efficiency. The presence of a small amount of bubbles will not interfere with performance).
Note: Samples can be stored overnight at –20°C.

Clean-up of Adapter Ligated DNA

51

Note

Allow AMPure XP Beads to warm to room temperature for at least 30 minutes before use.

52 Vortex AMPure Beads thoroughly to resuspend.

53 Add 57µl (~0.8X) of resuspended beads to the adaptor ligation reaction.

54 Mix well by vortexing for 3 – 5 seconds or by pipetting up and down 10 times.

55 Centrifuge briefly. Be sure to stop the centrifugation before the beads start to settle out.

56 Incubate samples on bench top for at least 5 minutes at room temperature.

57 Place the tube/plate on an appropriate magnetic stand for 5 minutes to separate the beads from the supernatant.

58 Carefully remove and discard the supernatant. Be careful not to disturb the beads that contain the DNA targets.

59 Incubate at room temperature for 30 seconds.

60 Carefully remove and discard the supernatant without disturbing the pellet.

61 Add 200µl of 80% freshly prepared ethanol to the tube/plate while in the magnetic stand. Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube/plate, place back on the magnet and remove traces of ethanol with a p10 pipette tip.

62 Air dry the beads for up to 5 minutes while the tube/plate is on the magnetic stand with the lid open.

Safety information

Do not over-dry the beads. This may result in lower recovery of DNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack, they are too dry.

63 Remove the tube/plate from the magnetic stand.

64 Add 17µl of nuclease free water to elute the DNA target from the beads.

65 Mix well on a vortex mixer or by pipetting up and down 10 times.

66 Incubate at room temperature for at 2 minutes.

67 Quickly spin to collect the liquid from the sides of the tube or plate wells.

68 Place the tubes/ plate on the magnetic stand and incubate for at least 5 minutes at room temperature.

69 Transfer 15µl (i.e. 7.5µl twice) of the supernatant to a new tubes/ plate for amplification.

Note

Samples can be stored at −20°C.

PCR Enrichment

70

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
NEBNext Ultra II Q5 Master Mix (blue top)	25
Universal PCR primer (blue top)	5

Total	30

Table 13. PCR Master Mix

Note

The PCR will take approximately 30 minutes

71 Add 30µl of the master mix into newly labelled tubes/ plate.

72 Add 15.0µl of adaptor-ligated DNA to the respective wells.

73 Mix well on a vortex mixer or by pipetting up and down 10 times.

74 Perform a quick spin to collect all liquid from the sides of the tube.

75 Place the tubes/plate on a thermocycler and perform PCR using the following conditions:

76

Temperature (°C)	Time	Cycles
98	3 minutes	1
98	10 seconds	8
60	30 seconds	
65	45 seconds	
65	5 minutes	1
4	Hold	

Table 13. PCR Conditions

Clean-up of Enrichment PCR

77

Note

If using AMPure XP Beads, allow the beads to warm to room temperature for at least 30 minutes before use

78 Vortex AMPure Beads thoroughly to resuspend.

79 Add 45µl (0.9X) resuspended AMPure beads to the PCR reaction.

80 Mix well by vortexing for 3 – 5 seconds or by pipetting up and down 10 times.

81 Centrifuge very briefly. Be sure to stop the centrifugation before the beads start to settle out.

82 Incubate samples on bench top for at least 5 minutes at room temperature.

83 Place the tube/plate on an appropriate magnetic stand for 5 minutes to separate the beads from the supernatant.

84 Carefully remove and discard the supernatant. Be careful not to disturb the beads that contain the DNA targets.

85 Add 200µl of 80% freshly prepared ethanol to the tube/plate while in the magnetic stand.

86 Incubate at room temperature for 30 seconds.

87 Carefully remove and discard the supernatant without disturbing the pellet.

88 Add 200µl of 80% freshly prepared ethanol to the tube/plate while in the magnetic stand. Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube/plate, place back on the magnet and remove traces of ethanol with a p10 pipette tip.

89 Air dry the beads for up to 5 minutes while the tube/plate is on the magnetic stand with the lid open.

Note

Do not over-dry the beads. This may result in lower recovery of DNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack, they are too dry.

90 Remove the tube/plate from the magnetic stand.

91 Add 33µl of nuclease free water to elute the DNA target from the beads.

92 Mix well on a vortex mixer or by pipetting up and down 10 times.

93 Quickly spin to collect the liquid from the sides of the tube or plate wells.

94 Incubate the tubes/ plate for 2 minutes at room temperature.

95 Place the tubes/ plate on the magnetic stand and incubate for at least 5 minutes.

96 Transfer 30µl (i.e. 15.5µl twice) of the supernatant to a new tubes/ plate.

97 Assess the concentration of the libraries using a Qubit fluorometer.

98 Assess the library fragments using the LabChip GX Touch.

Note

Samples can be stored at –20°C after clean-up.

Normalization of DNA

99 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

100 Pipette mix 5 times.

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

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

103 Proceed to library denaturation.

Library Denaturation

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

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

106 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.

107 Invert the tube several times to mix.

108 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 .

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

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

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

112 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.

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

Dilution of Denatured Library

114 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

115 Invert several times to mix and then pulse centrifuge.

116 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.

117 Combine the following volumes in a micro-centrifuge tube:

- 5 µL of 4 nM PhiX library
- 5 µL of 0.2 N NaOH

118 Vortex briefly to mix.

119 Centrifuge at 280 x g, 00:01:00 .

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

121 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

122 Invert to mix.

123 Combine Library and PhiX Control.

124 Mix this solution well and briefly centrifuge.

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

Citations

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