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Evaluation of miniaturized Illumina DNA preparation protocols for SARS-CoV-2 whole genome sequencing protocol

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Sureshnee Pillay[1], James Emmanuel San[1,2], Derek Tshiabuila[1], Yeshnee Naidoo[1,2], Yusasha Pillay[2], Akhil Maharaj[1], Ugochukwu J. Anyaneji[1,2], Eduan Wilkinson[2], Houriiyah Tegally[1,2], Richard J. Lessells[1,3], Cheryl Baxter[2,3], Tulio de Oliveira[1,2,3,4], Jennifer Giandhari[1]

[1]KwaZulu-Natal Research Innovation and Sequencing Platform (KRISP), Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa;
[2]Centre for Epidemic Response and Innovation (CERI), School of Data Science and Computational Thinking, Stellenbosch University, Stellenbosch, South Africa;
[3]Center for AIDS Programme of Research in South Africa (CAPRISA), Durban, South Africa;
[4]Department of Global Health, University of Washington, Seattle, WA, USA

KRISP

Sureshnee Pillay
UKZN

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Abstract

This protocol describes the miniaturisation of the Illumina DNA Prep library kit and the use of a rapid modified protocol with fewer clean-up steps and different reagent volumes (full volume, half volume, one-tenth volume). The cost of sequencing can be reduced roughly sevenfold, and the time required to prepare the library can be reduced from 6.5 hours to 3 hours. The protocol adaptation represents a lower-cost, streamlined approach for SARS-CoV-2 sequencing, which can be used to generate genomic data quickly and affordably, particularly in resource-constrained settings.

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 BiolabsCatalog #M0493S
Qubit™ Assay TubesInvitrogen - Thermo Fisher Catalog #Q32856
Qubit dsDNA HS Assay kit Thermo Fisher ScientificCatalog #Q32854
Artic Primers-specific for 2019-nCoV according to Primal Scheme Contributed by users
Nextera DNA Flex Library Prep Kit, Illumina, Inc.
Nextera™ DNA CD Indexes (96 Indexes 96 Samples) Contributed by users
MiSeq Reagent Nano Kit v2 (500 cycles)Illumina, Inc.Catalog #MS-103-1003
DNA High Sensitivity Reagent KitPerkin ElmerCatalog #CLS760672
DNA 1K / 12K / Hi Sensitivity Assay LabChipPerkin ElmerCatalog #760517
General PCR laboratory equipment and consumablesContributed by users

Troubleshooting

cDNA

1

Prepare the cDNA master mix in the pre-PCR clean room. The master mix 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 tube:

	A	B
	Component	**Volume (ul)**
	LunaScript	1
	Template RNA	5.5
	Total	6.5

Table 1. cDNA synthesis master-mix 1

2.1

Add 1 µl of LunaScript reagent into each well of a 96-well nested plate.

2.2

Transfer 5,5 ul of total nucleic acid extracts to each well as per Table 1.

2.3

The total volume in the tube should now be 6.5 µl.

3

Incubate the reaction as follows in a thermal cycler.

	A	B
	Temperature (°C)	**Time**

	A	B
	25 °C	2 minutes
	55 °C	10 minutes
	95 °C	1 minute
	4	Hold

Table 2. PCR conditions – cDNA synthesis

Primer Pool Preparation

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

5 To generate overlapping 400 nucleotide amplicons for this protocol, 2019-nCoV primers were designed using the Primal Scheme.

6 Resuspend lyophilised primers at a concentration of 100 M each if necessary.

7 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.

8 To make 10M primer stocks, dilute the 100M primer pool 1:10 in molecular-grade water. It is advised to make multiple aliquots of each primer pool in case of degradation or contamination.

Tiling PCR

9 Prepare the PCR mastermix in the clean mastermix room.

10

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

11

A master mix for each pool must be made up in the master mix hood.
Mix the following components in a labelled 1.5ml eppendorf tube:

	Component	Pool 1 volumes (µl)	Pool 2 volumes (µl)
	5X Q5 Reaction Buffer	2.5	2.5
	10mM dNTPs	0.25	0.25
	Q5 Hot Start DNA Polymerase	0.125	0.125
	Primer Pool 1 or 2 (10µM)	1.8	1.8
	Nuclease-free water	5.4	5.4
	Total	***10***	***10***

Table 3. PCR COVID-19 tiling PCR mastermix

11.1 Make a master mix by multiplying each component by the number of samples being tested.
Add 2.5 µl 5X Q5 Reaction Buffer to a labeled 1.5ml eppendorf tube.

11.2

Add 0.25 µl 10mM dNTPs.

11.3

Add 0.125 µl Q5 Hot Start DNA Polymerase.

11.4

Add 1.8 µl Primer Pool 1 or 2 (10µM).

11.5

Add 5.4 µl Nuclease-free water.

12

Aliquot 10 µl mastermix in labelled PCR strip tubes.

13

Add 2.5 µl of cDNA under the extraction hood or general lab hood, which has been decontaminated using 10% extran, and 70% ethanol, and sterilised with ultraviolet light (UV).

14

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

15

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 4. Tiling PCR conditions

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

16

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

Library Preparation: Tagmentation

17

Add 5 µl undiluted DNA to each well of a 96-well PCR plate / 0.2ml strip tubes.

18

	A	B	C
	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. Vortex to mix.

Table 5. Preparation of reagents

19

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

20

Vortex in between adding BLT as necessary.

21

Prepare the tagmentation master mix.

	A	B
	Component	**Volume (µl) per sample**
	BLT	1
	TB1	1
	Total	2

Table 6. Tagmentation Master Mix

21.1

Please scale this step as needed.

For 1 sample:

1 µl BLT
1 µl TB1
---
2 µl Total

22 Vortex the tagmentation master mix thoroughly.
Transfer 2 µl tagmentation master mix to each well of the plate containing sample.
Use fresh tips for each sample column.

23 Resuspend by pipetting each sample 10 times.

24 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

Table 7. PCR – Tagmentation conditions

Stop Tagmentation

25

	A	B	C
	Item	**Storage**	**Instructions**
	TSB (Tagment stop buffer)	15 oC to 30 oC	If precipitates are observed, heat at 37°C for 10 minutes, and then vortex until precipitates are dissolved. Use at room temperature.

Table 8. Preparation of Reagents

26 Add **1 µl TSB** to the tagmentation reaction.

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

28 Seal the plate with / tubes, place it on the preprogrammed thermal cycler, and run the stop tag programme.

29

	A	B
	Temperature (°C)	**Time**
	37	15 minutes
	10	Hold

Table 9. Stop-Tag programme

Post Tagmentation Clean

30 Place the plate on the magnetic stand for approximately **00:01:00** until liquid is clear.

31 Using a multichannel pipette, remove and discard supernatant.

32 Remove the sample plate from the magnetic stand and add 10 µl TWB directly onto the beads using a deliberately slow pipetting technique.
To avoid incorrect volume aspiration and incomplete mixing, this slow pipetting technique reduces the possibility of TWB foaming.

33 Slowly pipette until beads are fully resuspended.

34 Place the plate on the magnetic stand for approximately **00:01:00** until liquid is clear.

35 Using a multichannel pipette, remove and discard supernatant.

36 Remove the plate from the magnetic stand and add 10 µl TWB directly onto the beads using a deliberately slow pipetting technique.

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

38 Place the plate on the magnetic stand for approximately 00:01:00 until liquid is clear.

39 Using a multichannel pipette, remove and discard supernatant.

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

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

42 Seal the plate and keep on the magnetic stand until step 47 of the Procedure section in Amplify Tagmented DNA.

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

Amplify Tagment DNA

44

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

Table 10. Preparation of Reagents

45

	A	B
	Component	**Volume (µl) per sample**
	EPM	2
	NFW (Nuclease Free Water)	2
	Total	4

Table 11. PCR Master Mix

The following volumes were combined to prepare the PCR master mix (Table 13). Each volume was multiplied by the number of samples being processed. Reagent overage was included in the volume to ensure accurate pipetting.

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

47 With the plate on the magnetic stand, use a multichannel pipette to remove and discard the supernatant. (from step 42 of post-tagmentation clean-up).The foam that remains on the walls of the well does not adversely affect the library.

48 Remove from the magnet.

49 Immediately add 4 µl PCR master mix directly onto the beads in each sample well/ tube.

50 Pipette mix until the beads are fully resuspended. Alternatively, seal the plate and use a plate shaker at 1600 rpm for 00:01:00.

51 Seal the sample plate and centrifuge at 280 x g 00:00:30.

52 Add 1 µl of the appropriate index adapters to each sample.

53 Using a pipette set to 4 µl, pipette 10 times to mix. Alternatively, seal the plate/ tubes and use a plate shaker at 1600 rpm for 00:01:00.

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

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

56

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

Table 12. Enrichment PCR Conditions

57 **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

58

	A	B	C
	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.

	A	B	C
	RSB (Resuspension Buffer)	-25°C to-15°C	Thaw and bring to room temperature. Vortex to mix.

Table 13. Preparation of Reagents

59 Prepare fresh 80 % EtOH from absolute ethanol.

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

61 Place the plate/ tubes on a magnetic stand for approximately 00:01:00 until liquid is clear.

62 Transfer **4.5 µl** supernatant from each well of the PCR plate/ tubes to the corresponding well of a new plate/ tubes.

63 Add 4 µl nuclease-free water to each well/ tube.

64 Vortex and invert SPB multiple times to resuspend

65 Add 4.5 µl SPB to each well/ tube.

66 Mix well by pipetting 10 times. Alternatively, seal the plate and use a plate shaker at 1600 rpm for 00:01:00.

67 Incubate at Room temperature for 00:01:00.

68 Place on the magnetic stand for approximately 00:01:00 until the liquid is clear. During incubation, thoroughly vortex the SPB (undiluted stock tube), and then add 1.5 µl to each well of a new plate/tubes.

69 Transfer 12.5 µl supernatant from each well of the first plate/ tubes into the corresponding well of the second plate/ tubes (containing 1.5 µl undiluted SPB).

70 Mix well by pipetting 10 times. Alternatively, seal the plate and use a plate shaker at 1600 rpm for 00:01:00.

71 Discard the first plate/ tubes.

72 Pipette each well 10 times to mix.

73 Incubate at Room temperature for 00:01:00.

74 Place on the magnetic stand for approximately 00:01:00 until the liquid is clear.

75 Without disturbing the beads, remove and discard supernatant.

76 Wash two times as follows:

77 Add 20 µl freshly prepared 80% ethanol with the plate on the magnetic stand.

78 Incubate for 00:00:30.

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

80 Add 20 µl freshly prepared 80% ethanol with the plate on the magnetic stand.

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

82 Use a 20 µl pipette to remove any residual ethanol.

83 Air-dry on the magnetic stand for 00:01:00.

84 Remove from the magnetic stand.

85 Add 13.5 µl RSB to each well/ tube.

86 Resuspend by pipette mixing.

87 Incubate at Room temperature for 00:01:00.

88 Place the plate/ tubes on the magnetic stand for approximately 00:01:00.

89 Transfer 12 µl supernatant to a new 96-well PCR plate/ tubes.

90 **Rapid Method:**

Using an example of 96 samples:

Pool 4.5ul of each sample after the enrichment step into a 1.5 µl tube= 432 µl (4.5 × 96).

Add 384 µl (4× 96) of nuclease free water.

Add 432 µl (4.5 X96) of SPB.

Vortex and incubate for 5 minutes.

Place on amagnetic rack for 3 minutes.

Transfer the supernatant to a clean 1.5 µl tube.

Add 144 µl (1.5 × 96) of SPB.

Vortex and incubate for 5 minutes.

Place on a magnetic rack for 3 minutes.

Wash twice with 1 ml of 80% ethanol.

Remove all traces of ethanol after the last wash.

Elute in 35 µl of resuspension buffer.

91 **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

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

93 Calculate theappropriate amount of diluent on 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

94 Pipette mix 5 times.

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

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

97 Proceed to library denaturation.

Library Denaturation

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

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

100 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=1N1M=1N 10N(x)=(0.2)(500)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.

101 Invert the tube several times to mix.

102 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.

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

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

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

106 Add 990 µl of pre-chilled HT1 to the 10 µl of 4nM denatured library.
The result is a 20pM denatured library in 1 mM NaOH.

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

Dilution of Denatured Library

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

109 Dilute the denatured DNA to the desired concentration using the following example (if using 1% PhiX):

	Final Concentration	20pM denatured DNA	1% PhiX	Pre-chilledHT1

12 pM	354.6µl	3.6µl	240µl

Table 14. Dilution of denatured library to desired concentration

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

110 Invert several times to mix and then pulse centrifuge.

111 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, pH8.5 with 0.1 % Tween 20 If not prepared within the last 12 hours, prepare a fresh dilution of 0.2 N NaOH.

112 Combine the following volumes in a micro-centrifuge tube: • 5 µl of 4 nM PhiX library • 5 µl of 0.2 N NaOH

113 Vortex briefly to mix.

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

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

116 Dilute denatured PhiX to 20pM by adding pre-chilled HT1 to the denatured PhiX library as follows:
990 µl pre-chilled HT1 to 10 µl denatured PhiX library •

117 Invert to mix.

118 Combine Library and PhiX Control.

119 Mix this solution well and briefly centrifuge.

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