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nCoV-2019 Illumina Miniseq sequencing protocol (2,000bp amplicon)

Forked from nCoV-2019 sequencing protocol (RAPID barcoding, 1200bp amplicon)

DOI
https://dx.doi.org/10.17504/protocols.io.6qpvrdpjbgmk/v1

Bruno Gomez-Gil[1], Juli[2]

[1]CIAD AC Mazatlan; [2]CIAD

Juli : Julissa Enciso Ibarra;

Bruno Gomez-Gil
CIAD

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Protocol status: Working
We use this protocol and it's working

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Disclaimer

It work for us, any modification is up to you.

Abstract

This is a fork of the protocol https://dx.doi.org/10.17504/protocols.io.bh7hj9j6 but modified for tiled 2000bp amplicons, tagmentation with Nextera XT, indexing, and sequencing with the Illumina Miniseq platform.
It has already produced very good sequences.
Much of this protocol is base on this paper: https://doi.org/10.1093/biomethods/bpaa014

Guidelines

Tested with high viral copy numbers (<30 Ct).

Materials

cDNA

- **GoScript™ Reverse Transcriptase Kit** by Promega.

Multiplex DNA

-

Library preparation

- **Nextera XT** DNA Library Preparation Kit
-

Protocol materials

GoScript™ Reverse Transcriptase Kit **Promega** **Catalog #**A5001

KAPA Taq HotStart **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**KK1510

Nextera XT DNA Library Preparation Kit **Illumina, Inc.** **Catalog #**FC-131-1024

Safety warnings

Please follow standard health and safety guidelines when working with COVID-19 patient samples.

Sample preparation

10m

1 Dilute the sample depending on the Ct values, this will reduce the likelihood of PCR-inhibition.

Ct range	Sample	Water
12-15	2 µL	198 µL
16-18	2 µL	18 µL
>18	no dilution	

cDNA preparation

2h

2 We use the GoScript™ Reverse Transcriptase Kit **Promega Catalog #**A5001 5m

Mix (pipetting) the following components in Eppendorf tube.

Component	Volume
Nuclease-free water	4 µL
GoScriptTM Reaction Buffer, Random Primer	2 µL
GoScriptTM Enzyme Mix	0.4 µL
Final volume	10 µL

Note

It is good practice to carry a **negative control** (e.g. water) through the entire process from cDNA preparation to sequencing.

Note

The mastermix should be made up in the **mastermix cabinet** and aliquoted into PCR strip tubes. Tubes should be wiped down when entering and leaving the mastermix cabinet.

3 Prepare the **mastermix** on ice, mix by pipetting.

Component	Volume
GoScriptTM Reverse Transcription Mix	10 µL
RNA	3 µL
Final volume	13 µL

4 Incubate the reaction as follows: 1h 21m

Anneal primers 25 °C for 00:05:00
Extension 42 °C for 01:00:00
Inactivation 70 °C for 00:15:00

Snap cool in a prechilled metal rack or on ice 00:01:00

Note

A quick cooling step using a PCR cooling block or ice helps to inhibit secondary structure formation and can decrease variation in overall coverage.

Note

A mastermix should be made up in the **mastermix cabinet** and added to the denatured RNA in the **extraction and sample addition cabinet**. Tubes should be wiped down when entering and leaving the mastermix cabinet.

Primer pool preparation

5 **PRIMERS** for this protocol are described in protocol **nCoV-2019 sequencing protocol (RAPID barcoding, 1200bp amplicon)** and are the 2,000 bp option.
We selected for 2,000 bp amplicons so they could be more easily tagmented in the library preparation.
Two pooles are prepared, Pool1 with 18 primers and Pool2 with 16 primers.

POOL1. Average Tm 61.01 °C

	A	B	C	D	E	F	G	H	I	J	K	L
	Name	**Sequence**	**Direction**	**Start**	**End**	**Length**	**Product Size**	**Tm**	**%GC**	**Hairpin Tm**	**Pair Dimer Tm**	**Self Dimer Tm**
	SARSCoV_2000_01_LEFT	ACCAACCAACTTTCGATCTCTTGT	forward	31	54	24	2049	60.7	41.7	None	None	None
	SARSCoV_2000_01_RIGHT	ACACCACCTGTAATGTAGGCCA	reverse	2058	2079	22	2049	61.4	50	39	None	5
	SARSCoV_2000_03_LEFT	TCGCACAAATGTCTACTTAGCTGT	forward	3772	3795	24	1814	60.6	41.7	None	0.7	None
	SARSCoV_2000_03_RIGHT	GTGTGCCCATGTACATAACAGCT	reverse	5563	5585	23	1814	61.2	47.8	42.7	0.7	8.3
	SARSCoV_2000_05_LEFT	CAATCATGCAATTGTTTTTCAGCTATTTTG	forward	7299	7328	30	1825	60.4	30	33	None	7.4
	SARSCoV_2000_05_RIGHT	CGTGTGTCAGGGCGTAAACTTT	reverse	9102	9123	22	1825	61.6	50	None	None	None
	SARSCoV_2000_07_LEFT	GGACGTACCATATTGGGTAGTGC	forward	10886	10908	23	1834	60.8	52.2	43.8	None	None
	SARSCoV_2000_07_RIGHT	TCTGTCGTAGTGCAACAGGACT	reverse	12698	12719	22	1834	61.3	50	41.4	None	14
	SARSCoV_2000_09_LEFT	ACCACTTCAGAGAGCTAGGTGT	forward	14477	14498	22	1925	60.5	50	None	None	None
	SARSCoV_2000_09_RIGHT	ACAACCTGGAGCATTGCAAACA	reverse	16380	16401	22	1925	61.5	45.5	None	None	11.9

	A	B	C	D	E	F	G	H	I	J	K	L
	SARSCoV_2000_11_LEFT	TGGCATACCTAAGGACATGACCT	forward	18168	18190	23	1814	60.9	47.8	38.3	None	11.2
	SARSCoV_2000_11_RIGHT	CAGTGAGTGGTGCACAAATCGT	reverse	19960	19981	22	1814	61.6	50	38.7	None	20.4
	SARSCoV_2000_13_LEFT	TCCTCAGTTTTACATTCAACTCAGGA	forward	21695	21720	26	1937	60.2	38.5	45.2	None	0.9
	SARSCoV_2000_13_RIGHT	TGACTAGCTACACTACGTGCCC	reverse	23610	23631	22	1937	61.5	54.5	37	None	None
	SARSCoV_2000_15_LEFT	AGGAGTCAAATTACATTACACATAAACGAA	forward	25360	25389	30	1805	60.1	30	None	None	None
	SARSCoV_2000_15_RIGHT	ACTGCTACTGGAATGGTCTGTGT	reverse	27142	27164	23	1805	61.6	47.8	None	None	None
	SARSCoV_2000_17_LEFT	ACTTGTCACGCCTAAACGAACA	forward	27873	27894	22	1918	60.7	45.5	36.7	None	None
	SARSCoV_2000_17_RIGHT	TAGGCAGCTCTCCCTAGCATTG	reverse	29769	29790	22	1918	61.6	54.5	45.3	None	None

Pool1

POOL2. Average Tm 61.05 °C

	A	B	C	D	E	F	G	H	I	J	K	L
	Name	**Sequence**	**Direction**	**Start**	**End**	**Length**	**Product Size**	**Tm**	**%GC**	**Hairpin Tm**	**Pair Dimer Tm**	**Self Dimer Tm**

	A	B	C	D	E	F	G	H	I	J	K	L
	SARSCoV_2000_02_LEFT	AGGCCGCTATAACAATACTAGATGGA	forward	1956	1981	26	1923	61.3	42.3	None	None	None
	SARSCoV_2000_02_RIGHT	CAGCGATCTTTTGTTCAACTTGCT	reverse	3855	3878	24	1923	60.8	41.7	None	None	None
	SARSCoV_2000_04_LEFT	TCAACATGCCAATTTAGATTCTTGCA	forward	5473	5498	26	1929	60.3	34.6	None	8.1	None
	SARSCoV_2000_04_RIGHT	GCTGAAATCGGGGCCATTTGTA	reverse	7380	7401	22	1929	61.5	50	None	8.1	4.4
	SARSCoV_2000_06_LEFT	GCTGCTGAATGTACAATTTTTAAAGATGC	forward	9011	9039	29	2003	61.1	34.5	None	None	None
	SARSCoV_2000_06_RIGHT	AACCAGTGGTGTGTACCCTTGA	reverse	10992	11013	22	2003	61.5	50	47	None	17.6
	SARSCoV_2000_08_LEFT	TCACCTAATTTAGCATGGCCTCTT	forward	12620	12643	24	1956	60.1	41.7	None	None	3.3
	SARSCoV_2000_08_RIGHT	CAGGGTCAGCAGCATACACAAG	reverse	14554	14575	22	1956	61.5	54.5	None	None	None
	SARSCoV_2000_10_LEFT	TGCATACGTAGACCATTCTTATGTTGT	forward	16291	16317	27	1985	60.8	37	32.8	None	0.1
	SARSCoV_2000_10_RIGHT	GCTTCTTCGCGGGTGATAAACA	reverse	18254	18275	22	1985	61.5	50	None	None	6.1
	SARSCoV_2000_12_LEFT	GGACTACAAAAGAGATGCTCCAGC	forward	19878	19901	24	1920	61.5	50	42.5	11.2	None
	SARSCoV_200	ACCTCTTAGTACCAT	reverse	21775	21797	23	1920	60.5	47.8	37.1	11.2	12.7

	A	B	C	D	E	F	G	H	I	J	K	L
	0_12_RIGHT	TGGTCCCA										
	SARSCoV_2000_14_LEFT	GCTGAACATGTCAACAACTCATATGA	forward	23519	23544	26	1973	60.1	38.5	35.2	None	5.1
	SARSCoV_2000_14_RIGHT	TGCAGTAGCGCGAACAAAATCT	reverse	25470	25491	22	1973	61.4	45.5	46.2	None	12.1
	SARSCoV_2000_16_LEFT	TCTTATTACAAATTGGGAGCTTCGCA	forward	27051	27076	26	1995	61.3	38.5	37.6	None	None
	SARSCoV_2000_16_RIGHT	GCTTCTTAGAAGCCTCAGCAGC	reverse	29024	29045	22	1995	61.6	54.5	43.6	None	30.4

Pool2.

6 **PRIMER STOCKS** ([M] 100 micromolar (µM))

If you have ordered each primer independently and need to generate primer pool stocks: add 5 µL of each primer from Pool 1 to a 1.5 mL Eppendorf labeled “Pool 1 (100µM)” and each primer from Pool 2 to a 1.5 mL Eppendorf labelled “Pool 2 (100µM)”. These are your [M] 100 micromolar (µM) **stocks** of each primer pool.

Note

Primers should be diluted and pooled in the **mastermix cabinet** which should be cleaned with decontamination wipes and UV sterilised before and after use.

7 **WORKING PRIMERS** ([M] 10 micromolar (µM))

Dilute the primer stocks **1:10** in molecular grade water, to generate **10µM primer working stocks**. It is recommend that multiple aliquots of each primer pool are made to in case of degradation or contamination.

Note

Primers need to be used at a **final concentration of** [M] 0.015 micromolar (µM) **per primer**.

8 **POOLING OF PRIMERS**

Label two 1.5 mL Eppendorf tubes, one as **POOL1** and the another as **POOL2**.

8.1 Add 10 µL of each of the primers of **set 1** go to step #7 to the Eppendorf tube labelled **POOL1,** final concentration will be [M] 10 micromolar (µM) each

8.2 Add 10 µL of each of the primers of **set 2** go to step #7 to the Eppendorf tube labelled **POOL2,** final concentration will be [M] 10 micromolar (µM) each

Multiplex PCR

9 In the PCR hood set up the multiplex mastermix reaction as follows in 2 0.2mL PCR tubes. We use the

KAPA Taq HotStart **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**KK1510

Component	**Pool 1**	**Pool 2**	**Final Concentration**
5X Kapa HotStart Buffer	2.5 µL	2.5 µL	[M] 1 X
25 mM MgCl2 [M] 1.5 millimolar (mM)	0.75 µL	0.75 µL	
10 mM dNTPs [M] 0.2 millimolar (mM) each	0.25 µL	0.25 µL	
Primer Pool 1 **or** 2 (10µM ea) [M] 0.5 micromolar (µM)	0.6 µL	0.6 µL	
Kapa Taq HotStart Polymerase	0.1 µL	0.1 µL	[M] 0.5 U
Nuclease-free water	5.8 µL	5.8 µL	

Final mastermix volume	10.0 µL	10.0 µL

Note

A PCR mastermix for each pool should be made up in the **mastermix cabinet** and aliquoted into PCR strip tubes. Tubes should be wiped down when entering and leaving the mastermix cabinet.

10 In the **extraction and sample addition cabinet** add 2.5 µL cDNA to each tube and mix well by pipetting.
The final volume will be 12.5 µL

Note

The **extraction and sample addition cabinet** should should be cleaned with decontamination wipes and UV sterilised before and after use.

11 Pulse centrifuge the tubes to collect the contents at the bottom of the tube.

12 Set-up the following program on the thermal cycler: 5m 45s

Step	Temperature	Time	Cycles
Heat Activation	98 °C	00:00:30	1
Denaturation	95 °C	00:00:15	25-35
Annealing and Extension	60 °C	00:05:00	25-35
Hold	4 °C	Indefinite	1

Note

Cycle number should be 25 for Ct 18-21 up to a maximum of 35 cycles for Ct 35. We typically use 30 cycles.

Note

It is advisable to check each PCR amplicon by electrophoresis (1% agarose). MORE INFO

Expected result

Final concentrations of PCR products can range from ~20- 150ng/ul.

Pooling and PCR quantification

13 Amplicon quantification to make an equimolar mixture.

Note

At this stage, care should be taken with amplified PCR products. Only open tubes in a designated post-PCR workspace with equipment that is separate from areas where primers and mastermixes are handled.

13.1 Put 1 µL of each pool in a Nanodrop or similar spectometer and quantify the DNA concentration.

13.2 Label a 1.5 mL Eppendorf tube for each sample and make a **equimolar mix** with the two pools.
Calculate to achieve a final concentration of [M] 50 ng/ul

14 Quantify DNA using a **Qubit** or other method.
Quantification using Nanodrop is not recommended for a good estimation of the final pool.

Note

Alternatively, these amplicons can also be used for Illumina sequencing, such as found here: **x.doi.org/10.17504/protocols.io.betejeje**

We have found that performing an Ampure XP bead clean up at this stage does not improve performance. Therefore, it is not necessary to clean up the PCR reaction at this step.

14.1 Protocol

Protocol

NAME

DNA quantification using the Qubit fluorometer

CREATED BY

Nikki Freed

Preview

14.1.1 Prepare a mastermix of Qubit™ working solution for the required number of samples and standards. The Qubit dsDNA kit requires 2 standards for calibration (see note below).

Per sample:

Qubit® dsDNA HS Reagent 1 µL

Qubit® dsDNA HS Buffer 199 µL

Note

If you have already performed a calibration on the Qubit machine for the selected assay you can use the previous calibration stored on the machine. We recommend performing a new calibration for every sample batch but a same-day calibration would be fine to use for multiple batches.

To avoid any cross-contamination, we recommend that you remove the total amount of working solution required for your samples and standards from the working solution bottle and then add the required volume to the appropriate tubes instead of pipetting directly from the bottle to each tube.

14.1.2 Label the tube lids. Do not label the side of the tube as this could interfere with the sample reading.

Note

Use only thin-wall, clear, 0.5mL PCR tubes. Acceptable tubes include Qubit™ assay tubes (Cat. No. Q32856)

14.1.3 Aliquot Qubit™ working solution to each tube:

- standard tubes requires 190µL of Qubit™ working solution
- sample tubes require anywhere from 180–199µL (depending how much sample you wish to add).

The final volume in each tube must be 200µL once sample/standard has been added.

14.1.4 Add 10µL of standard to the appropriate tube.

14.1.5 Add 1–20µL of each user sample to the appropriate tube.

Note

If you are adding 1–2µL of sample, use a P-2 pipette for best results.

14.1.6 Mix each tube vigorously by vortexing for 3–5 seconds.

14.1.7 Allow all tubes to incubate at room temperature for 2 minutes, then proceed to “Read standards and samples”.

14.1.8 On the Home screen of the Qubit™ 3 Fluorometer, press DNA, then select 1X dsDNA HS as the assay type. The Read standards screen is displayed. Press Read Standards to proceed.

> Note
>
> If you have already performed a calibration for the selected assay, the instrument prompts you to choose between reading new standards and running samples using the previous calibration. **If you want to use the previous calibration, skip to step 12**. Otherwise, continue with step 9.

14.1.9 Insert the tube containing Standard #1 into the sample chamber, close the lid, then press Read standard. When the reading is complete (~3 seconds), remove Standard #1.

14.1.10 Insert the tube containing Standard #2 into the sample chamber, close the lid, then press Read standard. When the reading is complete, remove Standard #2.

14.1.11 The instrument displays the results on the Read standard screen. For information on interpreting the calibration results, refer to the Qubit™ Fluorometer User Guide, available for download at thermofisher.com/qubit.

14.1.12 Press Run samples.

14.1.13 On the assay screen, select the sample volume and units:
- Press the + or – buttons on the wheel, or anywhere on the wheel itself, to select the sample volume added to the assay tube (from 1–20µL).
- From the unit dropdown menu, select the units for the output sample concentration (in this case choose ng/µL).

14.1.14 Insert a sample tube into the sample chamber, close the lid, then press Read tube. When the reading is complete (~3 seconds), remove the sample tube.

14.1.15 **The top value (in large font) is the concentration of the original sample and the bottom value is the dilution concentration**. For information on interpreting the sample results, refer to the Qubit™ Fluorometer User Guide.

14.1.16 Repeat step 14 until all samples have been read.

14.1.17 Carefully **record all results** and store run file from the Qubit on a memory stick.

14.1.18 All negative controls should ideally be 'too low' to read on the Qubit machine, but MUST be < 1ng per ul. If your negative controls >1ng per ul, considerable contamination has occurred and you must redo previous steps.

Normalisation

15 Label a 0.2 mL PCR tube for each sample.

15.1 Adjust the amount of DNA in the tube to be 100 ng total per sample in 7.5 µL molecular grade water.

> Note
>
> For example if your PCR reaction is at least [M] 100 ng/µl add 1ul of the PCR reaction to 6.5ul of molecular grade water. Use 7.5ul of the negative control, even if there is no detectable DNA in the PCR reaction.

Tagmentation 7m

16 Nextera XT DNA Library Preparation Kit **Illumina, Inc. Catalog #**FC-131-1024 We have reduced the amount of reagents used per reaction.

16.1 Label a 1.5 mL Eppendorf tube for each sample and add in this order:

Component	**Volume**
1. Tagment DNA buffer (TD)	3.75 µL
2. cDNA [M] 2 ng/µl	3 µL

Pipette 10 times to mix

16.2 Add **Amplicon Tagment Mix** 0.5 µL and pipette 10 times to mix

16.3 Centrifuge at 280 x g, 20°C, 00:01:00

16.4 Incubate in thermal cycler 10m
55 °C for 00:10:00
10 °C indefenitly

16.5 Add 2 µL of Neutralize Tagment Buffer (NT).
Pipette 10 times to mix

16.6 Centrifuge at 280 x g, 20°C, 00:01:00

16.7 Incubate at Room temperature for 00:05:00 0 °C 5m

16.8 Preserve the samples at 4-8 °C until use.

Indexing 30m

17 La siguiente reacción requiere de la enzima polimerasa 2x Ampigene HS Taq Mix Catalog # ENZ-NUC101-0200 y de los sets de Nextera XT. Cada set contiene 96 combinaciones de TAGs con esto podemos alcanzar 384 muestras usando los Nextera XT Index Kit v2 (Sets A,B,C y D) Catalog # 20027213;20027214;20027215;20027216.

17.1 A cada tubo de reaccion agregar:

Component	Volume
Nuclease-free water	10 µL
2x Ampigene HS Taq Mix.	10 µL
Nextera XT index i5	1 µL
Nextera XT index i7	1 µL
ADN Tagmentado	3 µL

Final volume 25 µL

17.2 Centrifuge at 280 x g, 20°C, 00:01:00 1m

17.3 Set-up the following program on the thermal cycler: 4m 40s

Cover 100 °C

Step	Temperature	Time	Cycles
Heat Activation	72 °C	00:03:00	1
Denaturation	95 °C	00:00:30	1
	95 °C	00:00:10	
Annealing and Extension	55 °C	00:00:30	14
	72 °C	00:00:30	
Hold	4 °C	Indefinite	1

18

Purificación final y pooling

19 Al volumen que se tiene en el tubo agregar 0.8X de perlas magnéticas Ampure XP.

19.1 Mezclar y spin-down.

19.2 Llevar al magneto hasta formar el pellet y desechar el sobrenadante.

19.3 Agregar 150 uL de etanol 80% en posición contraria al pellet. Esperar 30 segundos. Desechar el etanol.

19.4 Retirar el exceso de etanol y secar las perlas por 5 min.

19.5 Resuspender las perlas en 26 uL de Agua libre de nucleasas.

Mix y spin down. Incubar por 5 min a TA.

19.6 Llevar al magneto hasta formar el pellet y transferir 25 uL del sobrenadante a un nuevo tubo previamente etiquetado.

19.7 Cuantificar 2 uL por Qubit HS y analizar los tamaños mediante electroforesis en gel de agarosa al 1.0%.

Secuenciacion de bibliotecas en plataforma Illumina Miniseq

20 A partir de la concentración en ng/uL determinada por Qubit y obtenido el tamaño aproximado del fragmento, llevar cada una de las librerias a una concetracion de 4 nM. Transferir 5 uL de cada librerías a 4 nM aun tubo previamente etiquetado para obtener el pool final.
Seguir el protocolo Library Denaturing and miniseq Sample Loading del kit