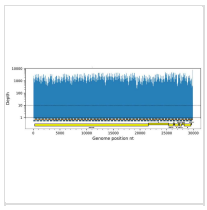

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Dec 06, 2021

nCoV-2019 sequencing protocol for illumina V.5

nCoV-2019 sequencing protocol

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protocol .

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This protocol was originally folked from "ARTIC amplicon sequencing protocol for MinION for nCoV-2019" by Josh Quick to adapt for any illumina sequencers (iSeq, MiSeq, NextSeq, etc.).

Because the PCR products are fragmented and ligated with adapters, this protocol is compatible to many sequencing kits with various read lengths (75PE, 150PE, etc.) for Illumina machines.

While the library preparation uses QIAseq FX by Qiagen and is basically straight forward (as par kit instruction but modified to 1/4 scale), some tweaks for much of simplicity and speed were added.

Change histories (V2):

1. The amount of cDNA input to multiplex PCR is increased (now almost same amount to the ARTIC Network's original protocol) (Step 10). This change gives better results for samples with extremely low RNA copy.
2. Amount of adapter solution input was corrected (Step 19).

Change histories (V3):

1. RT step has been changed to use of LunaScript (Tyson et al., 2020).
2. Corrected typos and wrong descriptions about amount of reagents.
3. Added a link to library quantification protocol.
4. Other minor changes.

Change histries (V4):

1. Added description about the primer set ver. N1/N2.
2. Added suggestion to double the concentration of nCoV-2019_74_LEFT and nCoV-2019_74_RIGHT.
3. Default annealing & extension temperature of multiplex PCR was changed to 64 °C.
4. Optional normalization step after PCR clean-up was omitted.

Change histries (V5):

1. Added description about a new primer set ver. N4 addressing drop-out in B.1.1.529 (Omicron).
2. Clean-up of each PCR products became optional. Instead, you can just dilute them and go.
3. PCR volume was reduced to 1/2.

Kentaro Itokawa, Tsuyoshi Sekizuka, Masanori Hashino, Rina Tanaka, Satsuki Eto, Risa Someno, Makoto Kuroda 2021. nCoV-2019 sequencing protocol for illumina. **protocols.io**
https://protocols.io/view/ncov-2019-sequencing-protocol-for-illumina-b2msqc6e
Kentaro Itokawa

nCoV-2019 sequencing protocol, Josh Quick

SARS-CoV-2, Genome Sequencing, illumina, B.1.1.529, B.1.617.2, PrimalSeq

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The important issue you have to consider first is how many samples you will multiplex in single run. This all depends on the capacity of your sequencer and the sample's viral loads represented by Ct-values in qPCR clinical test.

If a sample contains a relatively high copy number of virus genome (say, Ct < 25), the obtained reads usually distribute evenly across the genome. In such cases, only 10 Mb (330x) per sample is enough to cover the whole genome with sufficient coverage (>30x).

On the other hand, coverage bias increases as a sample's Ct-value increases. Hence, you will need more data to recover relatively weak regions. For samples containing only a low copy number of virus genome (Ct ~ 32), at least 100 Mb (3300x) is desirable to sequence the most part of the genome.

Also, if you are going to multiplex many samples in a low-throughput model (e.g. iSeq100), intensive normalization for each sample indix will be needed.

STEP MATERIALS

QIAseq FX DNA Library

Kit **Qiagen Catalog #180475** Step 13

QIAseq FX DNA Library

Kit **Qiagen Catalog #180475** Step 13

cDNA preparation

1

This protocol uses 5/8 reagents per sample compared to the original LunaScript protocol.

Mix the following components in an 0.2mL 8-strip tube or 96 well PCR plate;

Component	Volume
LunaScript RT SuperMix	**1.25 µL**
Template RNA (purified)	**5.0 µL**
Total	**6.25 µL**

A 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.

2 Gently mix by pipetting and pulse spin the tube to collect liquid at the bottom of the tube. 23m

Incubate the reaction as follows:

25 °C for **00:02:00**

55 °C for **00:20:00** (*)

95 °C for **00:01:00**

Hold at **4 °C**

*Incubation for 20 min on 55 °C is used instead of 10 min of the kit protocol to make sure cDNA is synthesized efficiently. However, we have not confirmed the benefit of this modification, yet.

Primer pool preparation (not everytimes)

3 If concentration of your primer stocks are 50 µM, generate primer pool stocks by adding each primers equally or as described in tables below to Eppendorf tubes labelled either “Pool 1 (50

μM)" or "Pool 2 (50 μM)". These are your 50 μM stocks of each primer pool.

If concentration of the primer stocks are 100 μM, dilute this primer pool **1:1** in molecular grade water, to generate 50 μM primer stocks.

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

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

4 *About primer set*

This protocol may be comaptible to any primer schemes (ARTIC Network V3/4, MidNight, NEB VerSkip, etc..).

N1 (NIID ver.1) primer set is a modified version of the ARTIC Network's V1 primer set. The N1 primer set includes replacement of 12 primers which involved in dimer formation with other primers (Itokawa et al., 2020).

Since December 2021, we updated the primer set to ver. N4. The modifications include additional primers addressing amplicon drop-out in two lineage of concern (VOC), B.1.617.2 (Delta) and B.1.1.529 (Omicron).

https://github.com/ItokawaK/Alt_nCov2019_primers/tree/master/Primers/ver_N4

Also, we confirmed that doubling the concentrations of nCoV-2019_64_LEFT, nCoV-2019_64_RIGHT, nCoV-2019_74_LEFT and nCoV-2019_74_RIGHT primers improves inherent low coverage of amplicon 64 and 74.

Here is the recommended formulation of N2 primer set.

Primer	Volume
nCoV-2019_1_LEFT	5 µL
nCoV-2019_1_RIGHTv2	5 µL
nCoV-2019_3_LEFT	5 µL
...	...
nCoV-2019_97_LEFT	5 µL
nCoV-2019_97_RIGHT	5 µL
nCoV-2019_73_LEFT_b11529b	**5 µL**
nCoV-2019_75_RIGHT_b11529a	**5 µL**
nCoV-2019_83_LEFT_b11529a	**5 µL**
Total	**505 µL**

Example of primer mixing for Pool1 of the N4 primer set

Primer	Volume
nCoV-2019_2_LEFT	5 µL
nCoV-2019_2_RIGHT	5 µL
...	...
nCoV-2019_64_LEFT	**10 µL**
nCoV-2019_64_RIHGT	**10 µL**
...	**...**
nCoV-2019_74_LEFT	**10 µL**
nCoV-2019_74_RIGHT	**10 µL**
...	...
nCoV-2019_98_LEFT	5 µL
nCoV-2019_98_RIGHT	5 µL
nCoV-2019_72_RIGHT_b16172a	**5 µL**
nCoV-2019_76_LEFT_b11529a	**5 µL**
nCoV-2019_92_LEFT_b11529a	**5 µL**
Total	**525 µL**

Example of primer mixing for Pool2 of the N4 primer set

Multiplex PCR

5 In the mastermix hood set up the multiplex PCR reactions as follows in 0.2 mL 8-strip PCR tubes or 96-well PCR plate:

Component	Pool 1	Pool 2
5X Q5 Reaction Buffer	**2.5 µL**	**2.5 µL**
dNTPs (**10 mM each**)	**0.25 µL**	**0.25 µL**
Q5 Hot Start DNA Polymerase	**0.125 µL**	**0.125 µL**

Primer Pool 1 or 2 (**50 µM**)	**0.36 µL**	**0.36 µL**
Nuclease-free water	**7.265 µL**	**7.265 µL**
Total	**10.5 µL**	**10.5 µL**

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.

If you use Q5 HS Hi-Fi 2X Master Mix, use 61-62 °C for Annealing & Extention temparature for following PCR.

6 In the **extraction and sample addition cabinet** add **2 µL** diluted cDNA to each tube and mix well by pipetting.

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

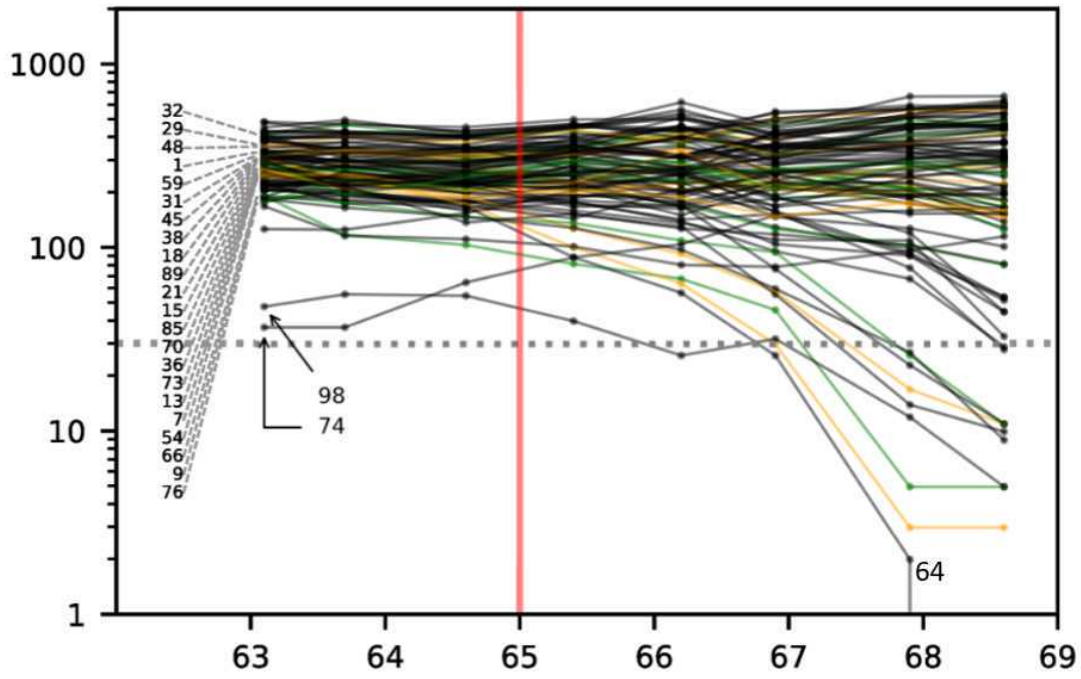
7 Pulse centrifuge the tubes to collect the contents at the bottom of the tube.

8 Set-up the following program on the thermal cycler:

Stage	Temperature	Time
Heat Activation	**98 °C**	**00:00:30**
30 cycles* of ...		
Denaturation	**98 °C**	**00:00:15**
Annealing	**64 °C** **	**00:05:00**
Hold	**4 °C**	Indefinite

*) We recommend 30 PCR cycles regardless of Ct-value because comparing PCR product yield and Ct-value gives a simple safeguard against sample swapping. However, one could increase this number to 35 to achieve more even PCR product yields.

) Previously, 65 °C was used for the annealing/extension step in multiplex PCR. However, inspecting many results in different thermocyclers and institutes, we now consider setting this temperature to **64 °C rather than 65 °C consistently yields better result with N1/N2 primer set. Nevertheless, fine-tuning of this temperature may still recommended to obtain the best result. Generally, amplicon 64 tends to show low coverage if annealing/extension temparature is higher than optimal.



Temperature and coverage of amplicons with N1 primer set and the protocol version 1 in TaKaRa Thermal Cycler Dice Touch. Modified from Itokawa et al., 2020 (CC BY 4.0).

PCR clean-up

9 (Option 1: With clean-up)

Combine each 10 µl contents of “Pool 1” and “Pool 2” PCR reactions for each biological sample into to a new 8-strip PCR tubes or 96-well PCR plate.

Clean-up the amplicons using the AmpureXP using 1x volume.

Elute in **20 µL** of low-TE buffer (10 mM tris-HCl pH8.0, 0.1 mM EDTA).

Transfer eluted DNA to a new 8-strip PCR tubes or 96-well PCR plate.

(Option 2: No clean-up)

Just combine each **2 µL** contents of “Pool 1” and “Pool 2” PCR reactions with **16 µL** milli-Q water (x5 dilution) for each biological sample.

Amplicon clean-up or dilution should be performed in the **post-PCR cabinet which** should should be cleaned with decontamination wipes and UV sterilised before and after use.

Quantification and normalilisation (optional)

10 Quantify the amplicon pools using fluorescent based method such as Qubit dsDNA HS kit (Invitrogen), Qaunt-it High-Sensitivity dsDNA Assay Kit (Invitrogen), or QuantiFluor® dsDNA System (Promega).

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* In our experience, samples with DNA concentration less than **2 ng / µL** in 30 cycles at this point do not generate meaningful results.

Fragmentation, End-prep & Adapter ligation

12

This protocol uses **1/4** reagents per sample compared to the original QIAseq FX DNA library kit (Qiagen: 180473 for 24-plex, 180475 and 180477 for 96-plex, and 180479, 180480, 180481 and 180482 for 384-plex).

Transfer **2 µL** of pooled & purified (diluted) PCR products to each well of a new 8-strip PCR tubes or 96-well PCR plate. Then, place the tubes or plate at 96 well aluminum block **On ice** .

Set the thermal cycler with a program below and start. Keep the heat-lid at **80 °C** .

4 °C pose

32 °C **00:06:00**

65 °C **00:30:00**

Prepare a master mix per one sample below.

Component	Volume / sample
FX Buffer, 10x	**1.25 µL**
H_2O	**6.75 µL**
FX Enzyme Mix	**2.5 µL**
Total	**10.5 µL**

Add **10.5 µL** of the above master mix to each well of the 8-strip PCR tubes or 96-well PCR plate. Mix well by pipetting.

Make sure the plate is always **On ice** during this procedure to avoid fragmentation reaction proceeds at this step.

Place the library plate into the thermal cycler posing at 4ºC and immediately skip to the next step (**32 °C**).

13 Remove the tube or plate from the thermal cycler after finishing the thermal program.

Take the adapter plate out of the kit box and thaw the content.

QIAseq FX DNA Library Kit **Qiagen Catalog #180475**

Keep both end-prepped DNA mixture and thawed adapter solution **On ice** during this step.

Add **1 µL** adapter solution to each end-prepped DNA mixture.

Prepare a master mix per sample below.

Component	Volume / sample
DNA Ligase Buffer, 5x	**5 µL**
DNA Ligase	**2.5 µL**
H_2O	**4 µL**
Total	**11.5 µL**

Add **11.5 µL** of above master mix to each end-prepped DNA mixture mixed with adapter **On ice** .

Set a thermal cycler with the following program with heat lid at **80 °C** .

1. **20 °C** **00:15:00**
2. **65 °C** **00:20:00** (ligase inactivation)

Start the thermal program, and place the tubes or plate immediately.

Seal and store remaining adapter plate in -20 °C. Extreme care should be taken to avoid cross-contamination between different indexed adapters.

Library pooling & purfication

14 Prepare a 1.5 or 2.0 ml low-binding tube.

Take **5 µL** * ligated mixture from each well and pool them into the 1.5 or 2.0 ml low-binding

tube.

*You can take different volumes by sample to adjust the DNA quantities for even representation of each index See the (**Guidelines & Warnings**). Use the DNA concentrations measured after the PCR clean-up.

Briefly measure the volume of pooled mixture in the 1.5 ml or 2.0 ml low-binding tube.

Purify by Ampure XP using **x0.8** volume.

Finaly, elute DNA in **50 µL** low-TE or Elution Buffer.

Transfer the eluted DNA to a new 1.5 or 2.0 ml low-binding tube.

Purify again by Ampure XP using **x1.2** volume.

Finaly, elute DNA in **25 µL** low-TE or Elution Buffer.

Transfer the eluted DNA to a new 1.5 or 2.0 ml low-binding tube.

Now, the library is ready for sequencing after quantification.

You may use any your routine methods for library quantification.

We recommend qPCR based methods in terms of accuracy and sensitivity.

Here is our homemade protocol.

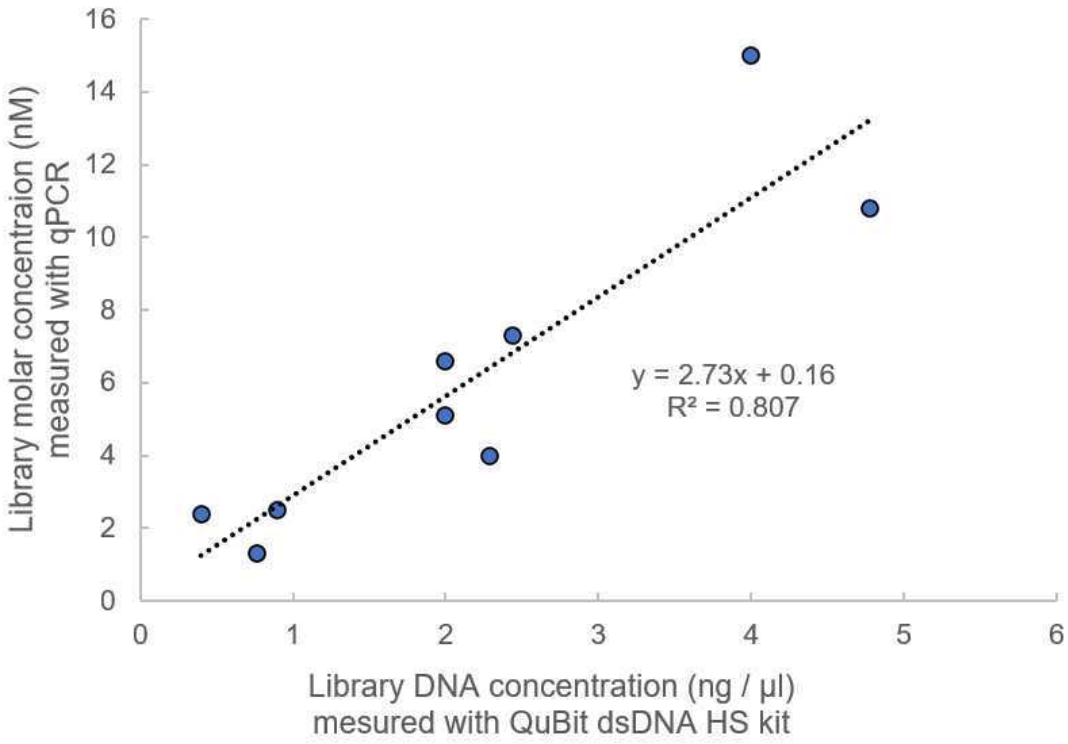
Illumina TruSeq Library quantification with qPCR probe method

by Kentaro Itokawa,

National Institute of Infectious Diseases, Japan

PREVIEW RUN

If you do not have a good quantification technique, here is a relationship between DNA mass concentration measured by Qubit dsDNA HS kit (Invitrogen) versus molar concentration measured by qPCR for nine libraries we obtained with this protocol.



Library DNA mass concentration measured by QuBit versus molar concentration measured by qPCR