Mar 30, 2020 Version 1

nCoV-2019 sequencing protocol for illumina V.1

Forked from nCoV-2019 sequencing protocol

DOI
dx.doi.org/10.17504/protocols.io.bd9fi93n

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Coronavirus Method De...

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OPEN ACCESS

DOI: dx.doi.org/10.17504/protocols.io.bd9fi93n

Protocol Citation: Kentaro Itokawa, Tsuyoshi Sekizuka, Masanori Hashino, Rina Tanaka, Makoto Kuroda 2020. nCoV-2019 sequencing protocol for illumina. **protocols.io https://dx.doi.org/10.17504/protocols.io.bd9fi93n**

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

Created: March 26, 2020

Last Modified: March 30, 2020

Protocol Integer ID: 34823

Keywords: SARS-CoV-2, Genome Sequencing, illumina,

Abstract

This protocol is folked from "**ARTIC amplicon sequencing protocol for MinION for nCoV-2019**" by Josh Quick to adapt it to **illumina sequencers**.

Because the PCR products are fragmented and ligated with adapters, this protocol is not restricted to 250 PE mode of MiSeq.

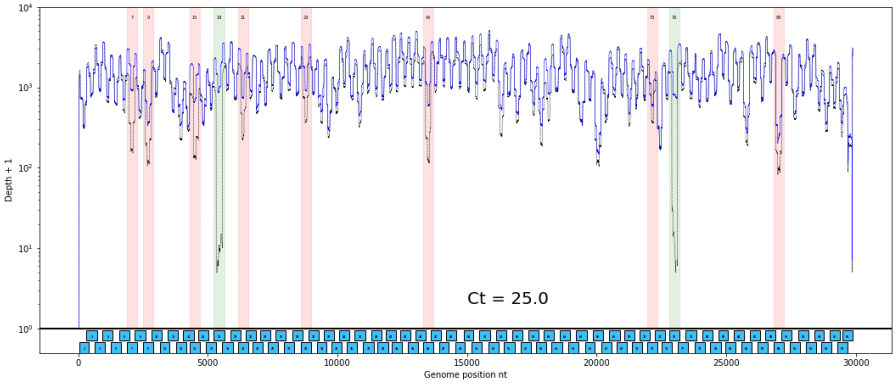
While the library preparation uses QiaSeq FX by Qiagen and is basically straight forward (as par kit instruction but set to 1/4 scale), some tweaks for much of simplicity and speed were added.

Guidelines

The important issue you have to consider first is how many samples you can multiplex in single run. This all depends on the capacity of a sequencer you have 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 good coverage.

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.



from https://www.biorxiv.org/content/10.1101/2020.03.10.985150v3

If you are going to multiplex a lot of samples using a low-throughput sequencer (e.g. iSeq100), intensive optimization for library concentrations of each sample will be needed.

Materials

STEP MATERIALS

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

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Protocol materials

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cDNA preparation

1

Note

This protocol uses 2/5 reagents per sample compared to the original ARTIC protocol.

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

Component	Volume
50µM random hexamers	0.25 µL
10mM dNTPs mix (10mM each)	0.25 µL
H_2O	0.5 µL
Template RNA	2.25 µL
Total	3.25 µL

Note

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.

3 Incubate the reaction as follows:

65 °C for 00:05:00

Place on ice for 00:01:00

4 Add the following to the annealed template RNA:

Component	Volume
SSIV Buffer	1 µL
100mM DTT	0.25 µL
RNaseOUT RNase Inhibitor	0.25 µL
SSIV Reverse Transcriptase	0.25 µL
Total	1.75 µL

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.

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

6 Incubate the reaction as follows:

42 °C 00:50:00
70 °C 00:10:00
Hold at 5 °C

Dilute cDNA by adding 20 µL H_2O to each reaction.

Primer pool preparation

7 If required resuspend lyophilised primers at a concentration of 100µM each

Note

nCov-2019/V1 primers for this protocol were designed using **Primal Scheme** and generate overlapping 400nt amplicons.

Note

We have proposed a modified primer set which gives better coverage than the original (V1) primer set. https://github.com/ItokawaK/Alt_nCov2019_primers
See detail described in the **preprint**.

8 Generate primer pool stocks by adding 5 µL of each primer pair to a 1.5 mL Eppendorf labelled either “Pool 1 (100µM)” or “Pool 2 (100µM)”. Total volume should be 490 µL for Pool 1 (100µM) and 490 µL for Pool 2 (100µM). These are your 100µM 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.

9 Dilute this primer pool 1:10 in molecular grade water, to generate 10µM primer 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 0.015µM per primer. In this case both pools have 98 primers in so the requirement is 3.6µL primer pools (10uM) per 25µL reaction. For other schemes, adjust the volume added appropriately.

Multiplex PCR

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

Component	Pool 1	Pool 2
5X Q5 Reaction Buffer	2 µL	2 µL
10 mM dNTPs	0.2 µL	0.2 µL
Q5 Hot Start DNA Polymerase	0.1 µL	0.1 µL
Primer Pool 1 or 2 (10µM)	1.44 µL	1.44 µL
Nuclease-free water	4.26 µL	4.26 µL
Total	8 µL	8 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.

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

Note

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

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

13 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	65 °C	00:05:00

Hold	4 °C	Indefinite

Note

! Use 30 PCR cycles regardless of Ct values.

PCR clean-up

14 Combine the entire 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.

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

Elute in 30 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.

Note

Amplicon clean-up 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 normalisation

16 Quantify the amplicon pools using the Quantus Fluorometer using Qubit dsDNA HS kit (Invitrogen).

17

!!! This step can be skipped if you prefer adjusting library concentrations after adapter ligation. We actually recommend normalizing library concentrations after adapter ligation since this is more immune to sample swapping. !!!

Normalize the input by diluting each sample to [M] 10 ng / µL by low-TE buffer.

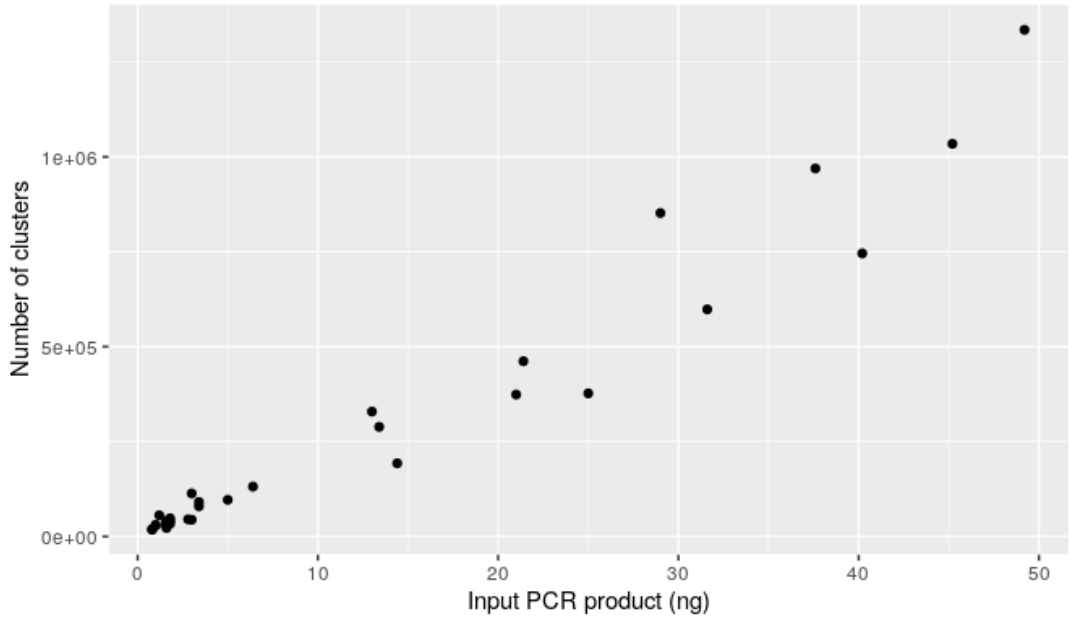
If concentration is less than [M] 10 ng / µL , dilution is not necessary *.

Note

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

Note

You can expect the quantity of DNA input will be directly proportional to the amount of data you will obtain if no further adjustment takes place.



One example for input DNA vs obtained clusters in single NGS run

Fragmentation, End-prep & Adapter ligation

18

Note

This protocol uses 1/4 reagents per sample compared to the original QIAseq FX DNA library kit.

Transfer 2 µL of PCR products (already combined for pool 1 and 2 and purified) 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

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

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.

Note

Make sure the plate is always On ice during this procedure to avoid fragmentation.

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

19 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

Note

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

Add 5 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	3.75 µL
Total	11.25 µL

Add 11.25 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 (inactivation)

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

Library pooling & purfication

20 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.

Note

*You can take different volumes by sample to adjust the DNA quantities of each library for optimal ratio.
See the **Guidelines & Warnings** section.

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.

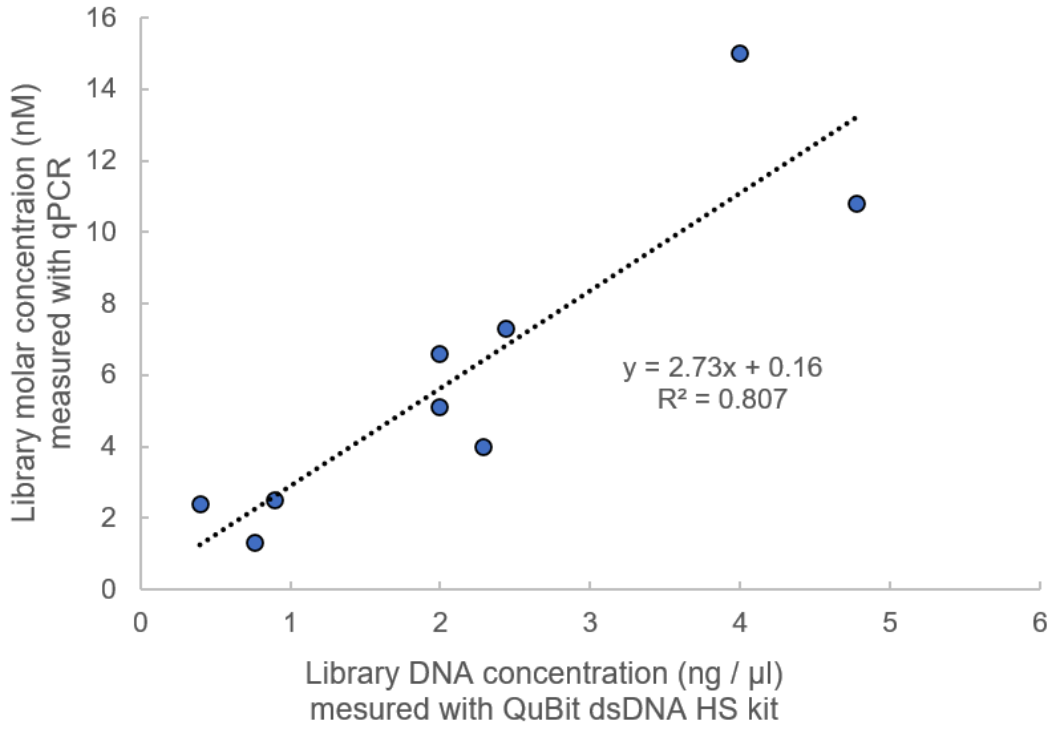
Transfer the eluted DNA to a new 1.5 or 2.0 ml low-binding tube.

Note

Now, the library is ready for sequencing after quantification.

You may use any routine methods working well for library quantification.
We recommend qPCR based methods in terms of accuracy and sensitivity.

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 recently we have obtained.



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