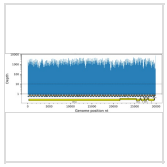

Version 3

Oct 22, 2020

nCoV-2019 sequencing protocol for illumina V.3

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1 Works for me dx.doi.org/10.17504/protocols.io.bnn7mdhn

Coronavirus Method Development Community

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ABSTRACT

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

In this Version V3, the reverse-transcription step was changed to use of NEB's LunaScript RT SuperMix Kit instead of Thermo Fisher's SuperScript IV Reverse Transcriptase as adapting to the recent update of ARTIC Network's protocol (LoCost).

Tyson et al., Improvements to the ARTIC multiplex PCR method for SARS-CoV-2 genome sequencing using nanopore
bioRxiv2020.09.04.283077;doi:https://doi.org/10.1101/2020.09.04.283077

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

DOI

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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.bnn7mdhn
Version created by Kentaro Itokawa

KEYWORDS

SARS-CoV-2, Genome Sequencing, illumina

CREATED

Oct 21, 2020

LAST MODIFIED

Oct 22, 2020

PROTOCOL INTEGER ID

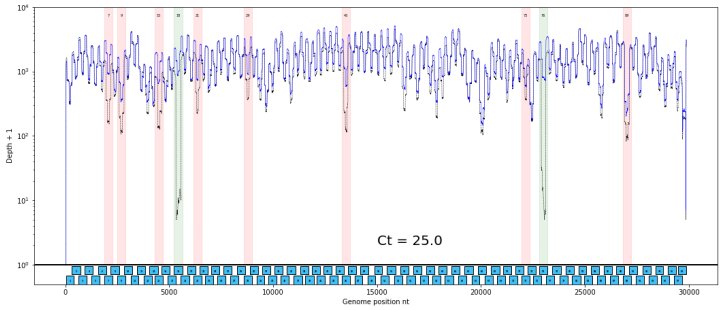
43455

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.

STEPS MATERIALS

NAME	CATALOG #	VENDOR
QIAseq FX DNA Library Kit	180475	Qiagen

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4. Other minor changes.

cDNA preparation

1

This protocol uses 5/16 reagents per sample compared to the original kit 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**

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

If concentration of the primer stocks are 50 µM, skip next step.

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 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 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 **0.72** µL primer pools (50 µM) per 25µL reaction. For other schemes, adjust the volume added appropriately.

Multiplex PCR

5 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	**5 µl**	**5 µl**
dNTPs (**10 mM each**)	**0.5 µl**	**0.5 µl**
Q5 Hot Start DNA Polymerase	**0.25 µl**	**0.25 µl**
Primer Pool 1 or 2 (**50 µM**)	**0.72 µl**	**0.72 µl**
Nuclease-free water	**16.53 µl**	**16.53 µl**
Total	**23 µl**	**23 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.

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

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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	**65 °C**	**00:05:00**
Hold	**4 °C**	Indefinite

We recommend 30 PCR cycles regardless of Ct values.

PCR clean-up

9 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.

10 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.

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 normalilisation (optional)

11 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).

12 **!!! 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**

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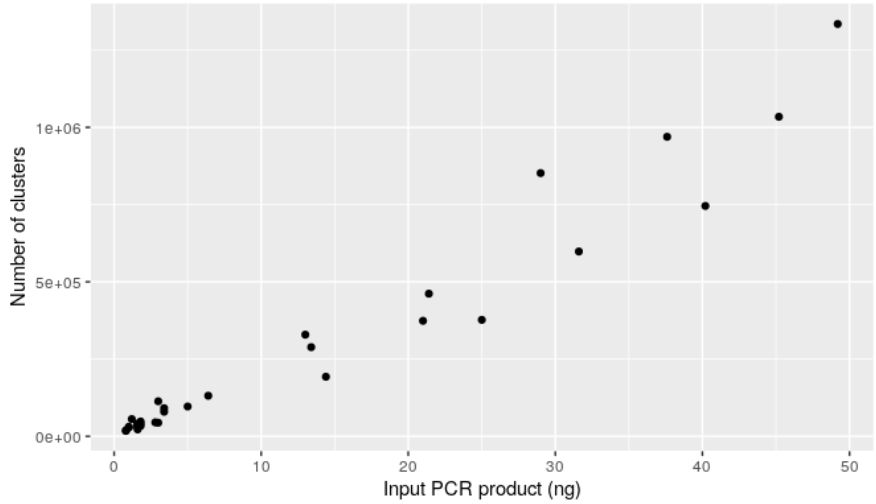
immune to sample swapping. !!!

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 *.

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

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.



An example of input DNA vs number of read-pairs in single NGS run

Fragmentation, End-prep & Adapter ligation

13

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

Transfer **2 µl** of pooled & purified 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** .

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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
by 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.

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**).

14 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
by Qiagen
Catalog #: 180475

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

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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** (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 indices.

Library pooling & purfication

15 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 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.

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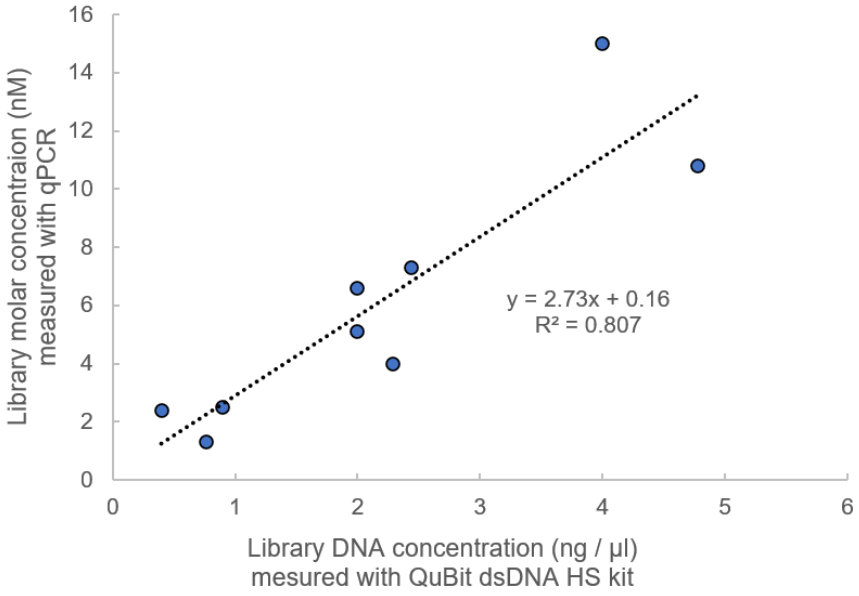
Now, the library is ready for sequencing after quantification.

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

Here is our homemade protocol.

Illumina TruSeq Library quantification with qPCR probe method PREVIEW RUN
by Kentaro Itokawa,
National Institute of Infectious Diseases, Japan

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 qPCR

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