Mar 14, 2020

nCoV-2019 sequencing protocol (single sample)

Forked from nCoV-2019 sequencing protocol

In 1 collection

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Coronavirus Method De...
1 more workspace

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

Created: March 06, 2020

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Protocol Integer ID: 33863

Abstract

ARTIC amplicon sequencing protocol for MinION for nCoV-2019

Materials

Primers lab-ready IDT https://github.com/artic-network/artic-ncov2019/tree/master/primer_schemes/nCoV-2019/V1

Extraction kits; Zymo Quick-RNA Viral Kit	Zymo	R1034 or
QIAamp Viral RNA Mini	Qiagen	52904
SuperScript IV (50 rxn)	Thermo	18090050
dNTP mix (10 mM each)	Thermo	R0192
Random Hexamers (50 µM)	Thermo	N8080127
RNase OUT (125 rxn)	Thermo	10777019
Q5 Hot Start HF Polymerase	NEB	M0493S
NEBNext Ultra II End-prep	NEB	E7546S
NEBNext Quick Ligation Module	NEB	E6056S
AMX, LNB, SFB, EB and SQB	Nanopore	SQK-LSK109
Flow Cell Priming Kit	Nanopore	EXP-FLP002
R9.4.1 flow cells	Nanopore	FLO-MIN106

cDNA preparation

1 Mix the following components in an 0.2mL 8-strip tube;

Component	Volume
50µM random hexamers	1 µL
10mM dNTPs mix (10mM each)	1 µL
Template RNA	11 µL
Total	13 µL

Note

Viral RNA input from a clinical sample should be between Ct 18-35. If Ct is between 12-15, then dilute the sample 100-fold in water, if between 15-18 then dilute 10-fold in water. This will reduce the likelihood of PCR-inhibition.

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	4 µL
100mM DTT	1 µL
RNaseOUT RNase Inhibitor	1 µL

SSIV Reverse Transcriptase	1 µL
Total	20 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

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

Note

nCov-2019/V2 primers for this protocol were designed using **Primal Scheme** and generate overlapping 400nt amplicons. Primer names and pools are listed in the .TSV file.

Primer pool preparation

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:

Component	**Pool 1**	**Pool 2**
5X Q5 Reaction Buffer	5 µL	5 µL
10 mM dNTPs	0.5 µL	0.5 µL
Q5 Hot Start DNA Polymerase	0.25 µL	0.25 µL
Primer Pool 1 or 2 (10µM)	3.6 µL	3.6 µL
Nuclease-free water	13.15 µL	13.15 µL
Total	22.5 µL	22.5 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 2.5 µL 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:

Step	**Temperature**	**Time**	**Cycles**
Heat Activation	98 °C	00:00:30	1
Denaturation	98 °C	00:00:15	25-35
Annealing	65 °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

PCR clean-up

14 Combine the entire contents of "Pool 1" and "Pool 2" PCR reactions for each biological sample into to a single 1.5 mL Eppendorf tube.

15 Clean-up the amplicons using the following protocol:

Protocol

NAME

Amplicon clean-up using SPRI beads

CREATED BY

Josh Quick

PREVIEW

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.

15.1 Vortex SPRI beads thoroughly to ensure they are well resuspended, the solution should be a homogenous brown colour. ⚠

Agencourt AMPure XP **Beckman Coulter** **Catalog #**A63880

15.2 Add an equal volume (1:1) of SPRI beads to the sample tube and mix gently by either flicking or pipetting. For example add 50 µL SPRI beads to a 50 µL reaction.

15.3 Pulse centrifuge to collect all liquid at the bottom of the tube.

15.4 Incubate for 00:05:00 at room temperature.

15.5 Place on magnetic rack and incubate for 00:02:00 or until the beads have pelleted and the supernatant is completely clear.

15.6 Carefully remove and discard the supernatant, being careful not to touch the bead pellet.

15.7 Add 200 µL of room-temperature [M] 70 % volume ethanol to the pellet.

15.8 Carefully remove and discard ethanol, being careful not to touch the bead pellet.

15.9 go to step #15.7 and repeat ethanol wash.

15.10 Pulse centrifuge to collect all liquid at the bottom of the tube and carefully remove as much residual ethanol as possible using a P10 pipette.

15.11 With the tube lid open incubate for 00:01:00 or until the pellet loses it's shine (if the pellet dries completely it will crack and become difficult to resuspend).

15.12 Resuspend pellet in 30 µL Elution Buffer (EB), mix gently by either flicking or pipetting and incubate for 00:02:00 .

Elution Buffer (EB) **Qiagen Catalog #**19086

15.13 Place on magnet and transfer sample to a clean 1.5mL Eppendorf tube ensuring no beads are transferred into this tube.

15.14 Quantify 1 µL product using the Quantus Fluorometer using the ONE dsDNA assay.

QuantiFluor(R) ONE dsDNA System, 100rxn **Promega Catalog #**E4871

Equipment

Quantus	NAME
Fluorometer	TYPE
Promega	BRAND
E6150	SKU
https://www.promega.co.uk/products/microplate-readers-fluorometers-luminometers/fluorometers/quantus-fluorometer	LINK

Quantification and normalisation

16 Quantify the amplicon pools using the Quantus Fluorometer using the ONE dsDNA assay.

Protocol

NAME

DNA quantification using the Quantus fluorometer

CREATED BY

Josh Quick

PREVIEW

Note

If the concentration is greater than 25 ng/µL dilute the sample by a factor of 10 by adding 270µL 10mM Tris and quantify again using the Quantus fluorometer.

16.1 Remove Lambda DNA 400 ng/µL standard from the freezer and leave on ice to thaw. Remove ONE dsDNA dye solution from the fridge and allow to come to room temperature.

QuantiFluor(R) ONE dsDNA System, 500rxn **Promega Catalog** #E4870

16.2 Set up two 0.5 mL tubes for the calibration and label them 'Blank' and 'Standard'

16.3 Add 200 µL ONE dsDNA Dye solution to each tube.

16.4 Mix the Lambda DNA standard 400 ng/µL standard by pipetting then add 1 µL to one of the standard tube.

16.5 Mix each sample vigorously by vortexing for 00:00:05 and pulse centrifuge to collect the liquid.

16.6 Allow both tubes to incubate at room temperature for 00:02:00 before proceeding.

16.7 Selection 'Calibrate' then 'ONE DNA' then place the blank sample in the reader then select 'Read Blank'. Now place the standard in the reader and select 'Read Std'.

16.8 Set up the required number of 0.5 mL tubes for the number of DNA samples to be quantified.

Note

Use only thin-wall, clear, 0.5mL PCR tubes such as Axygen #PCR-05-C

16.9 Label the tubes on the lids, avoid marking the sides of the tube as this could interfere with the sample reading.

16.10 Add 199 µL ONE dsDNA dye solution to each tube.

16.11 Add 1 µL of each user sample to the appropriate tube.

> **Note**
>
> Use a P2 pipette for highest accuracy.

16.12 Mix each sample vigorously by vortexing for 00:00:05 and pulse centrifuge to collect the liquid.

16.13 Allow all tubes to incubate at room temperature for 00:02:00 before proceeding.

16.14 On the Home screen of the Quantus Fluorometer, select `Protocol`, then select `ONE DNA` as the assay type.

> **Note**
>
> If you have already performed a calibration for the selected assay you can continue, there is no need to perform repeat calibrations when using ONE DNA pre diluted dye solution. If you want to use the previous calibration, skip to step 11. Otherwise, continue with step 9.

16.15 On the home screen navigate to 'Sample Volume' and set it to 1 µL then 'Units' and set it to ng/µL.

16.16 Load the first sample into the reader and close the lid. The sample concentration is automatically read when you close the lid.

16.17 Repeat step 16 until all samples have been read.

16.18 The value displayed on the screen is the dsDNA concentration in ng/µL, carefully record all results in a spreadsheet or laboratory notebook.

17 Label a 1.5 mL Eppendorf tube for each sample.

Note

This is a 'one-pot ligation' protocol for native barcoded ligation libraries. We have seen no reduction in performance compared to standard libraries, and is made faster by using the Ultra II® ligation module which is compatible with the Ultra II® end repair/dA-tailing module removing a clean-up step.

18 Normalise the input by diluting each sample to [M] 5 ng/µL. Use 10 µL input for the end-preparation reaction to give a total input of 50 ng

Note

Input to the one-pot native barcoding reaction will vary depending on the amplicon length but we have determined 5ng is the correct input for efficient barcoding of this amplicon length. Process at least 7 samples plus one negative control per native barcoded library in order to have sufficient material at the end.

End-preparation

19 Perform end-preparation on the single amplicon pool using the Ultra II End Repair/dA-Tailing module

Protocol

NAME

End-preparation of amplicon pools

CREATED BY

Josh Quick

PREVIEW

19.1 Set up the following reaction for each sample:

Component	Volume
DNA amplicons (5ng/ul)	10 µL
Nuclease-free water	2.5 µL
Ultra II End Prep Reaction Buffer	1.75 µL
Ultra II End Prep Enzyme Mix	0.75 µL
Total	15 µL

19.2 Incubate at room temperature for 00:05:00

Incubate at 65 °C for 00:05:00

Incubate on ice for 00:01:00

End-preparation clean-up

20 Clean-up end-preparation reaction using a 1x volume of SPRI beads and elute in 30 µL Elution Buffer (EB)

Protocol

NAME

Amplicon clean-up using SPRI beads

CREATED BY

Josh Quick

PREVIEW

20.1 Vortex SPRI beads thoroughly to ensure they are well resuspended, the solution should be a homogenous brown colour. ⚠

Agencourt AMPure XP **Beckman Coulter** **Catalog** #A63880

20.2 Add an equal volume (1:1) of SPRI beads to the sample tube and mix gently by either flicking or pipetting. For example add 50 µL SPRI beads to a 50 µL reaction.

20.3 Pulse centrifuge to collect all liquid at the bottom of the tube.

20.4 Incubate for 00:05:00 at room temperature.

20.5 Place on magnetic rack and incubate for 00:02:00 or until the beads have pelleted and the supernatant is completely clear.

20.6 Carefully remove and discard the supernatant, being careful not to touch the bead pellet.

20.7 Add 200 µL of room-temperature [M] 70 % volume ethanol to the pellet.

20.8 Carefully remove and discard ethanol, being careful not to touch the bead pellet.

20.9 go to step #15.7 and repeat ethanol wash.

20.10 Pulse centrifuge to collect all liquid at the bottom of the tube and carefully remove as much residual ethanol as possible using a P10 pipette.

20.11 With the tube lid open incubate for 00:01:00 or until the pellet loses it's shine (if the pellet dries completely it will crack and become difficult to resuspend).

20.12 Resuspend pellet in 30 µL Elution Buffer (EB), mix gently by either flicking or pipetting and incubate for 00:02:00 .

Elution Buffer (EB) **Qiagen Catalog #**19086

20.13 Place on magnet and transfer sample to a clean 1.5mL Eppendorf tube ensuring no beads are transferred into this tube.

20.14 Quantify 1 µL product using the Quantus Fluorometer using the ONE dsDNA assay.

QuantiFluor(R) ONE dsDNA System, 100rxn **Promega Catalog #**E4871

Equipment

Quantus	NAME
Fluorometer	TYPE
Promega	BRAND
E6150	SKU
https://www.promega.co.uk/products/microplate-readers-fluorometers-luminometers/fluorometers/quantus-fluorometer	LINK

AMX ligation

21 Perfom adapter ligation on the single amplicon pool with AMX.

Protocol

NAME

Adapter ligation with AMX

CREATED BY

Josh Quick

PREVIEW

21.1 Set up the following AMX adapter ligation reaction:

Component	Volume
End-repaired amplicon pools	30 µL
Ligation Buffer (LNB)	10 µL
Adapter Mix (AMX)	5 µL
Quick T4 DNA Ligase	5 µL
Total	50 µL

Note

There will be some variation in clean-up efficiencies but expect to carry around 80% through a clean-up.

21.2 Incubate at room temperature for 00:10:00

21.3 Add 50 µL (1:1) of SPRI beads to the sample tube and mix gently by either flicking or pipetting.

Note

Vortex SPRI beads thoroughly before use to ensure they are well resuspended, the solution should be a homogenous brown colour.

21.4 Pulse centrifuge to collect all liquid at the bottom of the tube.

21.5 Incubate for 00:05:00 at room temperature.

21.6 Place on magnetic rack and incubate for 00:02:00 or until the beads have pelleted and the supernatant is completely clear.

21.7 Carefully remove and discard the supernatant, being careful not to touch the bead pellet.

21.8 Add 250 µL SFB and resuspend beads completely by pipette mixing.

Note

SFB will remove excess adapter without damaging the adapter-protein complexes. Do not use 70% ethanol as in early clean-ups.

21.9 Pulse centrifuge to collect all liquid at the bottom of the tube.

21.10 Remove supernatant and discard.

21.11 Repeat steps 14-16 to perform a second SFB wash.

21.12 Pulse centrifuge and remove any residual SFB.

Note

You do not need to allow to air dry with SFB washes.

21.13 Add 15 µL EB and resuspend beads by pipette mixing.

21.14 Incubate at room temperature for 00:02:00 .

21.15 Place on magnetic rack.

21.16 Transfer final library to a new 1.5mL Eppendorf tube.

Library quantification

22 Quantify the final library using the Quantus Fluorometer using the ONE dsDNA assay.

Protocol

NAME

DNA quantification using the Quantus fluorometer

CREATED BY

Josh Quick

PREVIEW

Note

Final library can be now be stored in 10 mM Tris pH 8 at 4°C for up to a week if needed otherwise proceed directly to MinION sequencing.

22.1 Remove Lambda DNA 400 ng/µL standard from the freezer and leave on ice to thaw. Remove ONE dsDNA dye solution from the fridge and allow to come to room temperature.

QuantiFluor(R) ONE dsDNA System, 500rxn **Promega Catalog #**E4870

22.2 Set up two 0.5 mL tubes for the calibration and label them 'Blank' and 'Standard'

22.3 Add 200 µL ONE dsDNA Dye solution to each tube.

22.4 Mix the Lambda DNA standard 400 ng/µL standard by pipetting then add 1 µL to one of the standard tube.

22.5 Mix each sample vigorously by vortexing for 00:00:05 and pulse centrifuge to collect the liquid.

22.6 Allow both tubes to incubate at room temperature for 00:02:00 before proceeding.

22.7 Selection 'Calibrate' then 'ONE DNA' then place the blank sample in the reader then select 'Read Blank'. Now place the standard in the reader and select 'Read Std'.

22.8 Set up the required number of 0.5 mL tubes for the number of DNA samples to be quantified.

Note

Use only thin-wall, clear, 0.5mL PCR tubes such as Axygen #PCR-05-C

22.9 Label the tubes on the lids, avoid marking the sides of the tube as this could interfere with the sample reading.

22.10 Add 199 µL ONE dsDNA dye solution to each tube.

22.11 Add 1 µL of each user sample to the appropriate tube.

Note

Use a P2 pipette for highest accuracy.

22.12 Mix each sample vigorously by vortexing for 00:00:05 and pulse centrifuge to collect the liquid.

22.13 Allow all tubes to incubate at room temperature for 00:02:00 before proceeding.

22.14 On the Home screen of the Quantus Fluorometer, select `Protocol`, then select `ONE DNA` as the assay type.

Note

If you have already performed a calibration for the selected assay you can continue, there is no need to perform repeat calibrations when using ONE DNA pre diluted dye solution. If you want to use the previous calibration, skip to step 11. Otherwise, continue with step 9.

22.15 On the home screen navigate to 'Sample Volume' and set it to 1 µL then 'Units' and set it to ng/µL.

22.16 Load the first sample into the reader and close the lid. The sample concentration is automatically read when you close the lid.

22.17 Repeat step 16 until all samples have been read.

22.18 The value displayed on the screen is the dsDNA concentration in ng/µL, carefully record all results in a spreadsheet or laboratory notebook.

MinION sequencing

23 Prime the flowcell and load 20 ng sequencing library onto the flowcell.

Protocol

NAME

Priming and loading a MinION flowcell

CREATED BY

Josh Quick

PREVIEW

Note

From experience we know 20 ng is optimum loading input for short amplicons.

23.1 Thaw the following reagents at room temperature before placing on ice:

Sequencing buffer (SQB)
Loading beads (LB)
Flush buffer (FLB)
Flush tether (FLT)

23.2 Add 30 µL FLT to the FLB tube and mix well by vortexing.

23.3 If required place a new MinION flowcell onto the MinION by flipping open the lip and pushing one end of the flowcell under the clip and pushing down gently.

23.4 Rotate the inlet port cover clockwise by 90° so that the priming port is visible.

23.5 Take a P1000 pipette and tip and set the volume to 800 µL. Place the tip in the inlet port and holding perpendicularly to the plane of the flowell remove any air from the inlet port by turning the volume dial anti-clockwise.

Note

Be careful not to remove so much volume that air is introduced onto the rectangular array via the outlet.

23.6 Load 800 µL of FLB (plus FLT) into the flow cell via the inlet port, dispense slowly and smoothly trying to avoid the introduction of any air bubbles.

23.7 Wait for 00:05:00 .

23.8 Gently lift the SpotON cover to open the SpotON port.

23.9 Load another 200 µL of FLB (plus FLT) into the flow cell via the inlet port, this will initiate a siphon at the SpotON port to allow you to load the library dilution.

23.10 In a new tube prepare the library dilution for sequencing:

Component	**Volume**
SQB	37.5 µL
LB	25.5 µL
Final library	12 µL
Total	75 µL

Note

Mix LB immediately before use as they settle quickly.

Dilute library in EB if required.

23.11 Mix the prepared library gently by pipetting up and down just prior to loading.

23.12 Add the 75 µL library dilution to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop siphons into the port before adding the next.

23.13 Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the inlet port and close the MinION lid.

24 Start the sequencing run using MinKNOW.

Protocol

NAME

Starting a MinION sequencing run using MinKNOW

CREATED BY

Josh Quick

PREVIEW

24.1 If required plug the MinION into the computer and wait for the MinION and flowcell to ben detected.

24.2 Choose flow cell 'FLO-MIN106' from the drop-down menu.

24.3 Then select the flowcell so a tick appears.

24.4 Click the 'New Experiment' button in the bottom left of the screen.

24.5 On the New experiment popup screen, select the running parameters for your experiment from the individual tabs:

Experiment: Name the run in the experiment field, leave the sample field blank.

Kit: Selection: Select LSK109 as there is no option for native barcoding (NBD104).

Run Options: Set the run length to 6 hours (you can stop the run once sufficient data has been collected as determined using RAMPART).

Basecalling: Leave basecalling turned but select 'fast basecalling'.

Output: The number of files that MinKNOW will write to a single folder. By default this is set to 4000 but can be reduced to make RAMPART update more frequently.

Click 'Start run'.

24.6 Monitor the progress of the run using the MinKNOW interface.