



2020



Forked from Ebola virus sequencing protocol

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ARTIC





**ABSTRACT** 

ARTIC amplicon sequencing protocol for MinION for nCoV-2019

## cDNA preparation

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

Component	Volume
50μM random hexamers	<b>□</b> 1 μl
10mM dNTPs mix (10mM each)	<b>□1</b> μl
Template RNA	⊒11 µl
Total	<b>⊒</b> 13 μl



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



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.

- Gently mix by pipetting and pulse spin the tube to collect liquid at the bottom of the tube.
- Incubate the reaction as follows: 3

& 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



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:

## Primer pool preparation

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



<u>nCov-2019/V1</u> primers for this protocol were designed using <u>Primal Scheme</u> and generate overlapping 400nt amplicons. Primer names and dilutions are listed in the table below.

6 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.



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.

Primers need to be used at a final concentration of  $0.015\mu M$  per primer. In this case both pools have 98 primers in so the requirement is  $3.6\mu L$  primer pools (10uM) per  $25\mu L$  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:

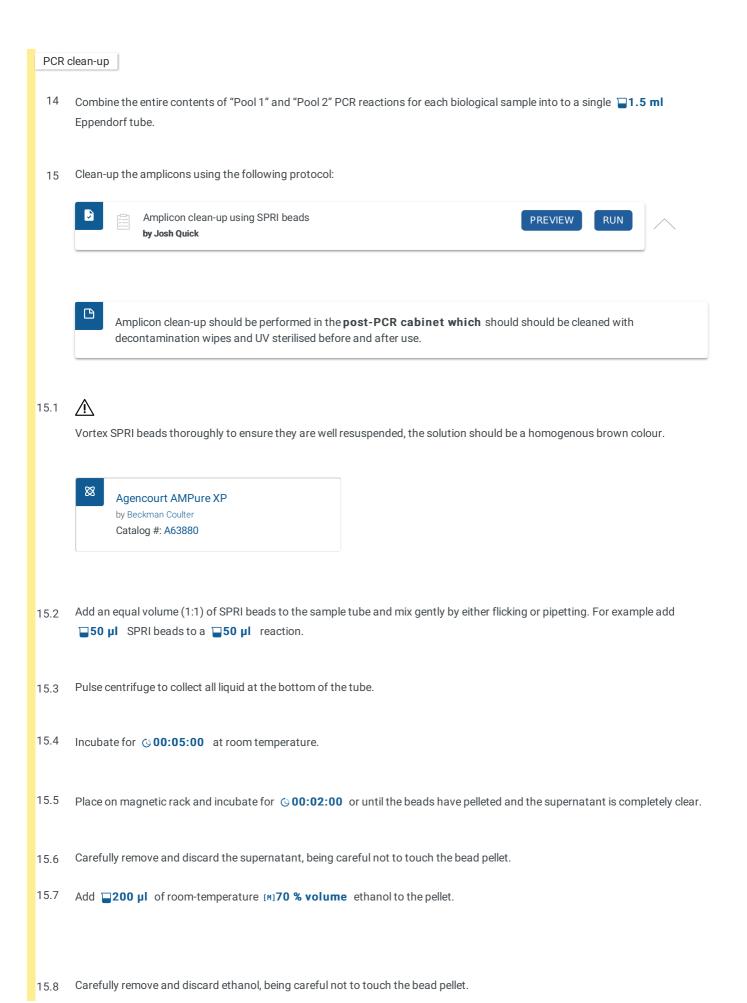
Component	Pool	1	Pool 2
5X Q5 Reaction Buffer	<b>⊒</b> 5 μl	<b>⊒</b> 5 μl	
10 mM dNTPs	<b>□</b> 0.5 μl	<b>□</b> 0.5 μl	
Q5 Hot Start DNA Polymerase	<b>⊒</b> 0.25 µl	□0.25	μl
Primer Pool 1 or 2 (10µM)	<b>⊒</b> 3.6 μl	<b>⊒</b> 3.6 μl	
Nuclease-free water	<b>□13.15</b> μl	<b>□</b> 13.15	μl
Total	<b>⊒22.5</b> μl	<b>⊒22.5</b> μl	

- A PCR
  - 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  $2.5 \, \mu$ l 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.
- 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	Tempe	rature Time		Cycles
Heat Activation	8 98 °C	© 00:00:30	1	
Denaturation	§ 98 ℃	<b>© 00:00:15</b>	25-35	
Annealing	8 65 °C	<b>© 00:05:00</b>	25-35	
Hold	8 4 °C	Indefinite	1	



Cycle number should be 25 for Ct 18-21 up to a maximum of 35 cycles for Ct 35



- 15.9 **ogo to step #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 🏿 Elution Buffer (EB), mix gently by either flicking or pipetting and incubate for 😗 00:02:00 .



- 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
    by Promega
    Catalog #: E4871
  - Quantus
    Fluorometer
    Promega E6150 ©

16.7

the reader and select 'Read Std'.

16.8	Set up the required number of <b>0.5 ml</b> tubes for the number of DNA samples to be quantified.	
	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.	
6.10	Add 199 μl ONE dsDNA dye solution to each tube.	
6.11	Add 🔲 1 µl of each user sample to the appropriate tube.	
	Use a P2 pipette for highest accuracy.	
6.12	Mix each sample vigorously by vortexing for $© 00:00:05$ and pulse centrifuge to collect the liquid.	
6.13	Allow all tubes to incubate at room temperature for <b>© 00:02:00</b> before proceeding.	
6.14	On the Home screen of the Quantus Fluorometer, select `Protocol`, then select `ONE DNA` as the assay type.	
	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.	
6.15	On the home screen navigate to 'Sample Volume' and set it to $\  \  \  \  \  \  \  \  \  \  \  \  \ $	
6.16	Load the first sample into the reader and close the lid. The sample concentration is automatically read when you close the lid.	
6.17	Repeat step 16 until all samples have been read.	
6.18	The value displayed on the screen is the dsDNA concentration in $ng/\mu L$ , carefully record all results in a spreadsheet or laboratory notebook.	

17 Label a 1.5 ml Eppendorf tube for each sample.



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.

Normalise the input by diluting each sample to [M]1 ng/µL. Use 5 µl input for the One-pot native barcoding reaction to give a total of 5 ng per sample.



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.

#### Native barocoding

19 Barcode the amplicon pools using native barcodes.



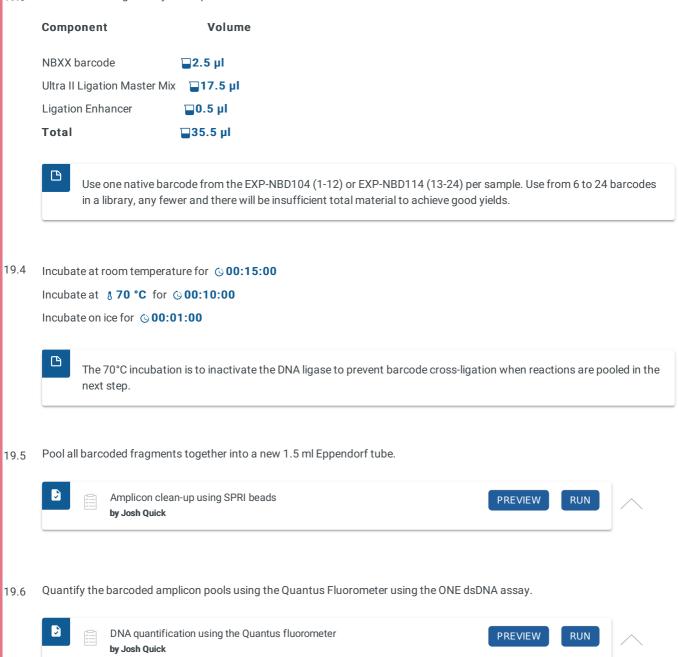
19.1 Set up the following reaction for each sample:

Component	Volume
DNA amplicons	<b>⊒</b> 5 μl
Nuclease-free water	<b>⊒</b> 7.5 μl
Ultra II End Prep Reaction Buffer	<b>□</b> 1.75 μl
Ultra II End Prep Enzyme Mix	<b>□</b> 0.75 µl
Total	<b>□</b> 15 μl

19.2 Incubate at room temperature for **© 00:10:00** 

Incubate at **§ 65 °C** for **© 00:05:00** 

#### 19.3 Add the following directly to the previous reactions:



19.7 Set up the following AMII adapter ligation reaction:

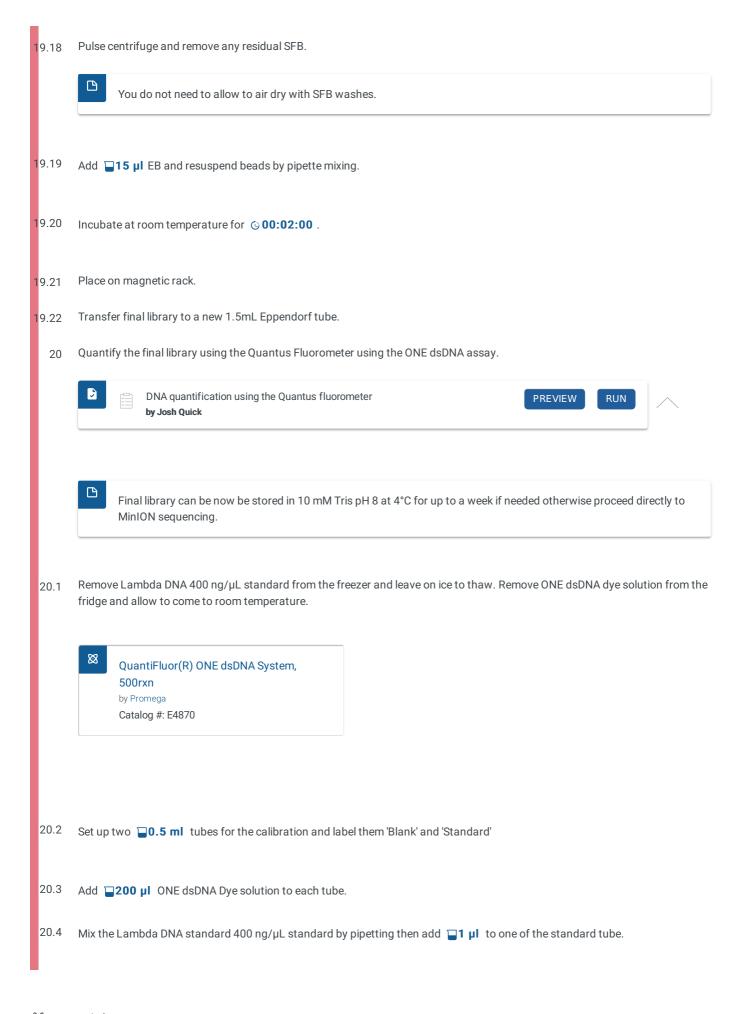
Remove supernatant and discard.

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

**1**9.16

**1**9.17

# **Volume** Component Barcoded amplicon pools **■30** µl NEBNext Quick Ligation Reaction Buffer (5X) **□**10 μl AMII adapter mix **■**5 μl Quick T4 DNA Ligase **□**5 μl Total **□**50 μl The input of barcoded amplicon pools will depend on the number of barcoded pools and should be between 40 ng (8 barcodes) and 160 ng (24 barcodes). 19.8 Incubate at room temperature for **© 00:15:00** 19.9 Add 50 µl (1:1) of SPRI beads to the sample tube and mix gently by either flicking or pipetting. Vortex SPRI beads thoroughly before use to ensure they are well resuspended, the solution should be a homogenous brown colour. Pulse centrifuge to collect all liquid at the bottom of the tube. 19.10 **1**9.11 Incubate for **© 00:05:00** at room temperature. 19.12 Place on magnetic rack and incubate for 600:02:00 or until the beads have pelleted and the supernatant is completely clear. 19.13 Carefully remove and discard the supernatant, being careful not to touch the bead pellet. 19.14 Add 200 µl SFB and resuspend beads completely by pipette mixing. ╚ SFB will remove excess adapter without damaging the adapter-protein complexes. Do not use 70% ethanol as in early clean-ups. Pulse centrifuge to collect all liquid at the bottom of the tube. 19.15



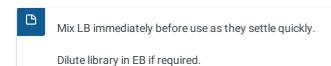
20.5 Mix each sample vigorously by vortexing for © 00:00:05 and pulse centrifuge to collect the liquid. 20.6 Allow both tubes to incubate at room temperature for © 00:02:00 before proceeding. Selection 'Calibrate' then 'ONE DNA' then place the blank sample in the reader then select 'Read Blank'. Now place the standard in 20.7 the reader and select 'Read Std'. 20.8 Set up the required number of **10.5 ml** tubes for the number of DNA samples to be quantified. Use only thin-wall, clear, 0.5mL PCR tubes such as Axygen #PCR-05-C Label the tubes on the lids, avoid marking the sides of the tube as this could interfere with the sample reading. 20.9 20.10 Add 199 µl ONE dsDNA dye solution to each tube. 20.11 Add  $\Box 1 \mu I$  of each user sample to the appropriate tube. Use a P2 pipette for highest accuracy. 20.12 Mix each sample vigorously by vortexing for  $\circlearrowleft 00:00:05$  and pulse centrifuge to collect the liquid. 20.13 Allow all tubes to incubate at room temperature for **© 00:02:00** before proceeding. On the Home screen of the Quantus Fluorometer, select 'Protocol', then select 'ONE DNA' as the assay type. 20 14 ₿ 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. 20.15 On the home screen navigate to 'Sample Volume' and set it to  $\[ \]$  1  $\mu$ I then 'Units' and set it to  $\[ \]$   $\eta$ L. Load the first sample into the reader and close the lid. The sample concentration is automatically read when you close the lid. 20.16 Repeat step 16 until all samples have been read. 20.17 The value displayed on the screen is the dsDNA concentration in ng/µL, carefully record all results in a spreadsheet or laboratory 20.18 notebook.

MinION sequencing 21 Prime the flowcell and load **20 ng** sequencing library onto the flowcell. Priming and loading a MinION flowcell **PREVIEW** RUN by Josh Quick From experience we know 20 ng is optimum loading input for short amplicons. Thaw the following reagents at room temperature before placing on ice: 21.1 Sequencing buffer (SQB) Loading beads (LB) Flush buffer (FLB) Flush tether (FLT) 21.2 Add 30 µl FLT to the FLB tube and mix well by vortexing. If required place a new MinION flowcell onto the MinION by flipping open the lip and pushing one end of the flowcell under the 21.3 clip and pushing down gently. Rotate the inlet port cover clockwise by 90° so that the priming port is visible. 21.4 21.5 Take a P1000 pipette and tip and set the volume to 300  $\mu$ I. 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. Be careful not to remove so much volume that air is introduced onto the rectangular array via the outlet. 21.6 Load 300 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. 21.7 Wait for **© 00:05:00** . Gently lift the SpotON cover to open the SpotON port. 21.8 21.9 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.

#### 21,10 In a new tube prepare the library dilution for sequencing:

Component	Volume
SQB	<b>⊒</b> 37.5 μl
LB	<b>⊒25.5</b> μl
Final library	<b>□12 μl</b>
Total	<b>⊒</b> 75 μl



- 21.11 Mix the prepared library gently by pipetting up and down just prior to loading.
- 21.12 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.
- 21.13 Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the inlet port and close the MinION lid.
  - 22 Start the sequencing run using MinKNOW.



- 22.1 If required plug the MinION into the computer and wait for the MinION and flowcell to ben detected.
- 22.2 Choose flow cell 'FLO-MIN106' from the drop-down menu.
- 22.3 Then select the flowcell so a tick appears.
- 22.4 Click the 'New Experiment' button in the bottom left of the screen.

22.5 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'.

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

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