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COVID-19 ARTIC v3 Illumina library construction and sequencing protocol V.4

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Coronavirus Method De... COG-UK

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We use this protocol and it's working

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Abstract

This SOP describes the procedure for generating cDNA from SARS-CoV-2 viral nucleic acid extracts and subsequently producing 400nt amplicons tiling the viral genome using V3 nCov-2019 primers (ARTIC). This is followed by library construction, equivolume pooling of samples and quantitation, prior to sequencing on the Illumina NovaSeq.

It is adapted from the nCov-2019 sequencing protocol which can be found here: https://www.protocols.io/view/ncov-2019-sequencing-protocol-bbmuik6w.

Guidelines

It is vital cDNA setup is performed in a laboratory in which post-PCR COVID-19 amplicons are not present, to minimise any risk of sample contamination.

Note: Throughout the protocol we have indicated the liquid handling automation in use at Sanger for specific parts of the process. However, these steps could be performed on alternative liquid handlers or manually.

Materials

MATERIALS

- NEBNext Ultra II DNA Library Prep Kit for Illumina - 96 rxns **New England Biolabs Catalog #**E7645L
- 2x Kapa HiFi Hotstart Readymix **Kapa Biosystems Catalog #**KK2602
- LunaScript RT SuperMix Kit **New England Biolabs Catalog #** E3010L
- Illumina Library Quantitation Complete kit (Universal) **Kapa Biosystems Catalog #**KK4824
- NEB Q5® Hot Start High-Fidelity 2X Master Mix **New England Biolabs Catalog #**M0494L
- AccuClear® Ultra High Sensitivity dsDNA Quantitation Kit with DNA Standards **Biotium Catalog ##**31028

STEP MATERIALS

- LunaScript RT SuperMix Kit **New England Biolabs Catalog #** E3010L
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Primer pool sequences (v3) can be found here:
https://github.com/joshquick/artic-ncov2019/blob/master/primer_schemes/nCoV-2019/V3/nCoV-2019.tsv

Protocol materials

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Troubleshooting

cDNA generation

1 **Important!** This step must be performed in a RNase free, pre-PCR environment in which post PCR COVID-19 amplicons are not present, to minimise risk of sample contamination.

Decontaminate bench surfaces, pipettes and gloves with RNase ZAP before starting work. Keep reagents and samples chilled throughout the process.

2 Defrost PCR plate containing 10 µL extracted RNA On ice .

3 LunaScript RT SuperMix Kit **New England Biolabs Catalog #** E3010L

Prepare RT mastermix in a dedicated UV treated pre-PCR area to minimise contamination risk.

	RT Master Mix	Vol / RXN (µL)	Vol/96 RXN (µL) inc. excess
	LunaScript Super Mix	4	461
	Nuclease-free water	6	691
	Total	**10**	**1152**

Mix thoroughly by vortexing.

4 Use the SPT Labtech Dragonfly Discovery to dispense 10 µL of RT mastermix into the PCR plate containing 10 µL extracted RNA.

5 Seal and briefly centrifuge plate.

6 Place plate on a thermocycler and run the following program:

	Temperature	Time
	25°C	2 minutes
	55°C	20 minutes

	95°C	1 minute
	4°C	∞
	Lid temp: Tracking	

7 **PAUSE POINT** cDNA can be stored at 4°C (same day) or -20°C (up to a week).

cDNA amplification

8

Note

Primer pool sequences (v3) can be found here: https://github.com/joshquick/artic-ncov2019/blob/master/primer_schemes/nCoV-2019/V3/nCoV-2019.tsv

NEB Q5® Hot Start High-Fidelity 2X Master Mix **New England Biolabs Catalog #**M0494L

Prepare the following mastermixes:

	PCR Primer Pool 1 Master Mix	**Vol/PCR RXN (µl)**	**Vol/ 96 plate (µl) inc. excess**
	Q5 Hotstart 2X Master Mix	12.5	1440
	Primer **Pool 1** (11µM total)	3.6	415
	Nuclease-free water	2.9	334
	Total	**19**	**2189**

Final concentration of each primer in the reaction is 0.015µM

	PCR Primer Pool 2 Master Mix	Vol/PCR RXN (µl)	Vol/ 96 plate (µl) inc. excess
	Q5 Hotstart 2X Master Mix	12.5	1440
	Primer **Pool 2** (10.8µM total)	3.6	415
	Nuclease-free water	2.9	334
	Total	**19**	**2189**

Final concentration of each primer in the reaction is 0.015µM

Mix thoroughly by vortexing.

9 Use the SPT Labtech Dragonfly Discovery to dispense 19 µL mastermix per well into 2×96 well plates.

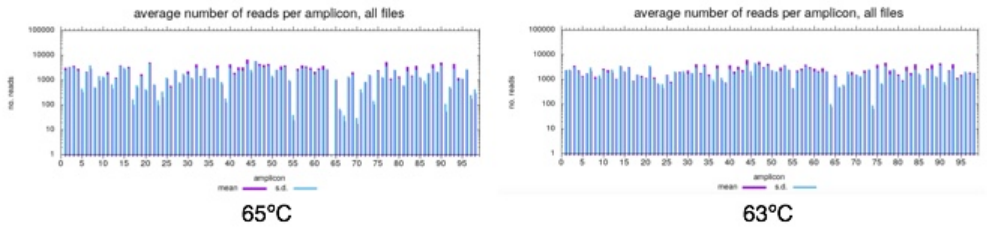
10 Use the Agilent Bravo to add 6 µL of cDNA template to each primer pool reaction and mix.

11 Heat seal and place the plates onto a thermocycler and run the following program. **Important!** Heat seal to minimise evaporation.

Note: Amplification should ideally be performed in a different lab to minimise the risk of contaminating other samples.

Expected result

Critical step: We strongly recommend performing a gradient PCR to determine the optimal annealing temperature for your thermocycler. Subtle differences in thermocycler calibration can result in specific amplicons dropping out. Reducing our annealing temperature from 65°C to 63°C for identical cDNA input recovered amplicon #64 as shown in the image below.



	Step	Temperature	Time
	1	98°C	30 seconds
	2	95°C	15 seconds
	3	63°C	5 minutes
	4	Repeat steps 2 & 3 for a total of 35 cycles	
	5	4°C	∞

12 **PAUSE POINT** Amplified cDNA can be stored at 4°C (overnight) or -20°C (up to a week).

Amplified cDNA SPRI

13 Allow AMPure XP beads to equilibrate to room temperature (~30 minutes). Ensure solution is homogenous prior to use, mixing gently by inversion.

14 Centrifuge amplified cDNA plates. 1000 x g, 00:01:00

15 Use either the Agilent Bravo or Beckman Coulter NX with a 96 well multichannel head to perform the following steps:

15.1 Combine the entire volumes of primer 1 and primer 2 PCR reactions per sample into one PCR plate.

15.2 Add **0.8X** volume of SPRI beads per sample (40 µL SPRI : 50 µL amplified cDNA), mix well by pipetting.

15.3 Incubate for 00:05:00 at Room temperature

15.4 Transfer the plate to the magnet, allow 00:02:00 for the beads to settle.

15.5 Carefully remove and discard the supernatant without disturbing the bead pellet.

15.6 Wash the beads with 180 µL 75% freshly prepared ethanol for 00:00:30 , then remove ethanol and discard.
(First wash)

15.7 Wash the beads with 180 µL 75% freshly prepared ethanol for 00:00:30 , then remove ethanol and discard.
(Second wash)

15.8 Allow beads to dry 00:05:00

15.9 Remove plate from magnet, add 20 µL nuclease-free water and resuspend by mixing well.

15.10 Incubate for 00:03:00 at Room temperature

15.11 Transfer the plate to the magnet, allow 00:05:00 for the beads to settle.

15.12 Carefully transfer supernatant into a new plate, taking care not to disturb the bead pellet.

16 **PAUSE POINT** Purified amplified cDNA can be stored at -20°C for several weeks prior to library preparation.

Amplified cDNA quantification

17

Note

Purified amplified cDNA is quantified with a fluorescence based assay. We use the **AccuClear Ultra High Sensitivity dsDNA Quantitation kit with 7 DNA standards** (Biotium) according to manufacturer's instructions.

To streamline the workflow, we do not normalise sample input for library preparation. Instead we confirm samples are in the range of **50ng-1ug** per **20µl sample** and take the entire volume into library preparation.

AccuClear® Ultra High Sensitivity dsDNA Quantitation Kit with DNA Standards **Biotium Catalog** ##31028

18 Pipette 20 µL of each DNA standard into wells A1 – G1 of a PCR plate. Add nuclease-free water to H1.

19 Dilute the AccuClear dye (100X) to working concentration by mixing 300 µL dye with 30 mL AccuClear buffer in a 50ml Falcon. Mix thoroughly by vortexing and transfer to a 384 well reservoir.

20 Use the SPT Labtech Mosquito LV to stamp 200nl of amplified cDNA and 1µl of known standards in triplicate into a 384 assay plate. Immediately proceed to the next step.

21 Use the Agilent Bravo 384ST to add 50 µL 1X AccuClear dye from the reservoir to the assay plate, mix thoroughly by pipetting.

22 Measure fluorescence values on a BMG FLUOstar Omega plate reader calibrated for use with AccuClear dye.

23 Confirm known standards are performing as expected.

24 Dilute any samples >125ng/µl with nuclease free water so they are in the range of 10 - 125ng/µl and repeat quantitation.

Note

Note: We use **5X** the volume of standard vs sample in our assay setup, which should allow a quantitative range of 0.15ng/µl – 125ng/µl. This deviates from the standard kit SOP which has a stated range of 0.03ng/µl – 25ng/µl.

25 Ensure all samples (20µl total volume) are in the range of 2.5-50ng/µl prior to proceeding with library preparation.

Library preparation for Illumina sequencing

26

Note

We use the NEB NEBNext® Ultra™ II DNA Library Prep Kit for Illumina, which we have automated on the Agilent Bravo platform with some modifications. 200ng is our standard input for library preparation, an acceptable range is 50ng – 1ug per sample. We use a custom adapter set, however any TruSeq adapters are suitable.

NEBNext Ultra II DNA Library Prep Kit for Illumina - 96 rxns **New England Biolabs Catalog #**E7645L

27

Prepare end prep mastermix On ice :

	NEBNext End Prep	Vol/PCR RXN (µl)	Vol/96 plate (µl) inc. excess

	NEBNext Ultra II End Prep Enzyme Mix	1.2	144
	NEBNext Ultra II End Prep Reaction Buffer	2.8	336
	Total	**4**	**480**

Mix well by pipetting.

28 The Bravo will combine 4 µL of end prep mastermix with 20 µL amplified cDNA and mix by pipetting.

29 Seal and transfer the plate to a thermocycler and run the following program:

	Temperature	**Time**
	20°C	30 minutes
	65°C	30 minutes
	4°C	∞

30 Prepare adapter ligation mastermix On ice :

	Adapter Ligation	**Vol/PCR RXN (µl)**	**Vol/96 plate (µl) inc. excess**
	NEBNext Ultra II Ligation Master Mix	12	1440
	NEBNext Ligation Enhancer	0.4	48
	TruSeq adapter (10µM)	1	120
	Total	**13.4**	**1608**

Mix well by pipetting.

31 The Bravo will add 13.4 µL adapter ligation mastermix to each sample and mix by pipetting.

32 The plate is incubated on deck at 20 °C for 00:15:00, however this step may also be performed on a thermocycler.

33

Note

Note: We use alternative TruSeq compatible adapters, which do not require the USER enzyme incubation step. If using NEBNext adapters, follow the steps in the NEB protocol to add USER enzyme to the ligation reaction.

34 A **0.8X** SPRI is performed post-ligation.

Ensure AMPure XP beads have been equilibrated to room temperature (~30 minutes) and the solution is homogenous prior to use.

The Bravo will perform a **0.8X** SPRI clean-up and elute sample in 25 µl nuclease-free water as follows:

34.1 Add 0.8X volume of SPRI beads per sample, mix well by pipetting.

34.2 Incubate for 00:05:00 at Room temperature.

34.3 Transfer the plate to the magnet, allow 00:02:00 for the beads to settle.

34.4 Carefully remove and discard the supernatant without disturbing the bead pellet.

34.5 Wash the beads with 180 µL 75% freshly prepared ethanol for 00:00:30, then remove ethanol and discard.
(First wash)

34.6 Wash the beads with 180 µL 75% freshly prepared ethanol for 00:00:30, then remove ethanol and discard.
(Second wash)

34.7 Allow beads to dry for 00:05:00

34.8 Remove plate from magnet, add 25 µL nuclease free water and resuspend by mixing well.

34.9 Incubate for 00:03:00 at Room temperature .

34.10 Transfer the plate to the magnet, allow 00:05:00 for the beads to settle.

34.11 Carefully transfer supernatant into a new plate, taking care not to disturb the bead pellet. Half of this eluate **(12.5µl)** is used as input for library PCR.

Library PCR

35

Note

We use KAPA HiFi HotStart ReadyMix and unique dual indexed (UDI) tag plates for library PCR.
Note: this deviates from the standard NEB protocol which uses NEBNext Ultra II Q5 Master Mix and different cycling conditions.

2x Kapa HiFi Hotstart Readymix **Kapa Biosystems Catalog #**KK2602

Prepare PCR mastermix On ice :

	KAPA PCR Mastermix	**Vol/PCR RXN (µl)**	**Vol/96 plate (µl) inc. excess**
	KAPA HiFi HotStart ReadyMix	25	3000
	Water	12.5	1500
	Total	**37.5**	**4500**

Mix well by pipetting.

36 The Bravo will add 37.5 µL PCR mastermix and 12.5 µL sample into a lyophilised plate of UDIs and mix thoroughly by pipetting. The final concentration of each UDI in the PCR reaction is 2µM.

37 Seal and transfer the plate to a thermocycler and run the following program:

	Temperature	Time
	95°C	5 minutes
	98°C	30 seconds
	65°C	30 seconds
	72°C	2 minutes
	Repeat 4 times	
	72°C	5 minutes
	4°C	∞

Construct equivolume pool

38 In a post-PCR lab, combine **5µl** of each sample per plate to form an equivolume pool of 96 samples.

38.1 Using a multichannel pipette, transfer 5 µL of each sample in the PCR plate into a low volume reservoir.

38.2 Transfer the contents of the reservoir into an Eppendorf tube and mix well.

Equivolume pool SPRI

39 Allow AMPure XP beads to equilibrate to room temperature (~30 minutes). Ensure solution is homogenous prior to use, mixing gently by inversion.

39.1 Add **0.8X** volume of SPRI beads per pool tube, mix well by pipetting.

39.2 Incubate for 00:05:00 at Room temperature .

39.3 Transfer the tube to a magnet, allow 00:05:00 for the beads to form a pellet.

39.4 Carefully remove and discard the supernatant, taking care not to disturb the bead pellet.

39.5 Wash the beads with 1 mL 75% ethanol for 00:00:30 then carefully remove ethanol and discard.
(First wash)

39.6 Wash the beads with 1 mL 75% ethanol for 00:00:30 then carefully remove ethanol and discard.
(Second wash)

39.7 Allow beads to dry for 00:05:00 .

39.8 Remove tube from magnet and resuspend beads in 1 mL elution buffer, mix well by pipetting.

39.9 Incubate for 00:03:00 at Room temperature

39.10 Transfer tube to magnet, allow 00:05:00 for the beads to form a pellet.

39.11 Carefully transfer supernatant into a new tube, taking care not to disturb the bead pellet.

Equivolume pool quantification

40

Note

Equivolume pools may be quantified either by qPCR or on an Agilent Bioanalyzer. Pools are then diluted to 1nM for sequencing.

qPCR
Quantify samples in triplicate using the KAPA Complete kit (Universal) for Illumina (KK4824) plus the KAPA Library Quantification Dilution Control (KK4906).

We use the SPT Labtech Mosquito LV to stamp library pools in triplicate into a 384 assay plate, and the Agilent Bravo to setup the qPCR reactions (1:1600 dilution).

qPCR is performed on the Roche LightCycler 480.

Agilent Bioanalyzer
Prepare 3 dilutions of the equivolume pool (1:10, 1:100, 1:1000). Run 1µl of each dilution in triplicate using the High Sensitivity DNA assay kit.

Confirm size distribution is as expected, check there is no primer-dimer or adapter-dimer present.

Sequencing

41

Note

We currently sequence samples on an Illumina NovaSeq SP flow cell, using the XP workflow.

Alternatively, samples may be sequenced on an Illumina MiSeq using either v2 (500 cycle) or v3 (600 cycle) reagent kits. We have plexed up to 96 samples per run, this could be increased further depending on coverage requirements. Loading concentration will need to be optimised for MiSeq.
MiSeq run parameters: Read length 212 paired end + 16bp.

42 The following protocol is for loading a NovaSeq. We currently plex up to 384 samples per NovaSeq SP lane.

43 Steps must be performed within a given timeframe or data quality may be affected. Therefore, ensure the instrument is washed, waste containers emptied and ready for use prior to beginning step 46.

44 Defrost Illumina NovaSeq SP SBS and cluster reagent cartridges for 2-4 hours in a Room temperature water bath. Use a lint free tissue to blot any water present on the foil seal. Gently mix cartridges 10X by inversion. Gently tap the bottom of the cartridges on the bench to reduce air bubbles.

45 Defrost components DPX1, DPX2 and DPX3 from a NovaSeq XP-2 lane kit, then keep On ice

46 Bring flow cell to Room temperature (~10 minutes) prior to use.

47 18 µL of each [M] 1 nanomolar (nM) pool is required per SP lane.
Denature pools by adding 4 µL 0.2N NaOH per 18µl. Vortex briefly to mix.

48 Incubate at Room temperature for 00:08:00

49 Add 5 µL 400mM Tris-HCl, pH8.0 to each tube to neutralise the reaction. Vortex briefly to mix, then keep On ice .

Note

For the following steps, keep samples and mastermix On ice until ready for loading onto the flow cell.

50 **Important!** Use mastermix within 01:00:00 of preparation for optimal sequencing performance.

Prepare ExAmp mastermix on ice:

	ExAmp Master Mix	**Volu**

		me per SP flow cell (µl)
	DPX1	126
	DPX2	18
	DPX3	66
	Total	**210**

Vortex 00:00:30 to mix, then centrifuge briefly up to 280 x g

51 Add 63 µL ExAmp mastermix to each denatured pool, mix well by pipetting.

52 Prepare the flowcell for sample loading by placing into the flow cell dock with the 2-lane manifold clamped in place.

53 Pipette 80 µL of library + ExAmp pool mix per manifold well. Wait for approximately 2 minutes to allow the solution to fill the lane.

54 **Important!** The sequencing run must be started within 00:30:00 of libraries being loaded onto the flow cell.

54.1 Unclamp the flow cell dock and discard the manifold. Load the flow cell onto the NovaSeq flow cell stage.

54.2 Load the SBS and cluster reagent cartridges.

54.3 Start sequencing run (250PE).